Analyzing genetic factors contributing to dysmotility in Hirschsprung's disease and African Degenerative Leiomyopathy in a South African population

by

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Declaration

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Abstract

Introduction

Hirschsprung's disease (HSCR) and African degenerative leiomyopathy (ADL) are rare gastrointestinal disorders affecting neonates and young children. HSCR is characterised by the absence of intrinsic ganglion cells in the distal segment of the intestine; its aetiology has been linked to cellular and molecular mechanisms associated with the enteric nervous system (ENS) development, ADL on the other hand is a distinctive form of visceral myopathy (VSCM) of uncertain aetiology affecting enteric smooth muscles (ESM) of the distal intestine. Gut motility is a result of highly coordinated contractions by muscle layers, neural network and pacemaker intestinal cells of Cajal whose development are controlled by genetic factors.

The aetiology of HSCR has been associated with 15 genes linked to ENS development meanwhile ADL has been linked to environmental factors. Actin gamma 2 (*ACTG2*) is a gene that encodes the ACTG2 protein which is involved in ESM development. Studying the *ACTG2* in HSCR patients may ascertain whether individuals affected by HSCR also display muscular dysfunction thereby providing a possible factor in the recurrence of dysmotility post-surgical resection. Additionally, *ACTG2* has been identified as the genetic factor in VSCM pathology; therefore, the study may provide novel information regarding the genetic factors of ADL.

Aim

This project aims to study the genes associated with the development of enteric nervous system (*RET, NRG1, SOX10, EDNRB*) and smooth muscle cells (*ACTG2*) that contribute to HSCR and ADL in the South African neonate population.

Methods

Seventeen whole blood samples were collected from HSCR participants after informed consent; of which only 14 samples were included for genotyping and 9 samples were selected for RNA analysis based on the quality of extracted DNA and RNA respectively. Five whole blood samples were also collected from ADL patients after informed consent. RNA samples from the HSCR cohort were reverse transcribed and quantitative polymerase chain reaction

was performed. DNA samples from HSCR and ADL samples were screened for variants in the *ACTG2* exons through bidirectional Sanger sequencing. Novel variants were analysed *in silico* to ascertain their pathogenicity.

Results and Discussion

In both HSCR and ADL cohorts the variant K119E/R was observed in 64% (9/14) and 60% (3/5) of the study population respectively; K119E/R is likely to function as a disease modifier as it was also observed in the control samples six out nine individuals. Variants S345L and W357G in exon 10 with probable significant effect in the pathogenesis of ESM were identified in the HSCR cohort only. The ADL cohort had polymorphic intronic variants predicted to shift the exonic splice sites namely g>c -IVS12 exon 3 and c>t -IVS3 exon 5. Differential expression of ENS genes *EDNRB*, *RET*, *SOX10* and *NRG1* associated with ENS development in the HSCR cohort was not achieved due to experimental factors.

Conclusion

ACTG2 encodes an enteric smooth muscle γ-2 actin which plays a pivotal role in the contractile proteins of ESM, thus the data suggests that a muscular component may exist in HSCR aetiology that should be investigated further *in vitro* and provides further insights into genetic factors that may contribute to ADL pathogenesis.

Opsomming

Inlieding

Hirschsprung se siekte (HSCR) en Afrika-degeneratiewe leiomyopatie (ADL) is skaars gastro-intestinale afwykings wat neonate en jong kinders raak. HSCR word gekenmerk deur die afwesigheid van intrinsieke ganglion selle in die distale segment van die dunderm; sy etiologie is gekoppel aan sellulêre en molekulêre meganismes wat verband hou met die ontwikkeling van die enteriese senuweestelsel (ENS). ADL, aan die ander kant, is 'n kenmerkende vorm van viscerale myopatie (VSCM) van onseker etiologie wat enteriese gladdespiere (ESM) van die distale dunderm beïnvloed . Gutmotiliteit is 'n gevolg van hoogs gekoördineerde kontraksies deur spierlae, neurale netwerk en pacemaker-intestinale selle van Cajal wie se ontwikkeling deur genetiese faktore beheer word.

Die etiologie van HSCR is geassosieer met 15 gene wat verband hou met ENS-ontwikkeling, terwyl ADL gekoppel is aan omgewingsfaktore. Actin gamma 2 (ACTG2) is 'n geen wat kodeer vir die ACTG2 proteïen wat betrokke is by ESM ontwikkeling. Die studie van die *ACTG2* in HSCR pasiënte kan vasstel of individue wat deur HSCR geraak word ook spierafwykings toon en sodoende 'n moontlike faktor in die herhaling van dysmotiliteit post-chirurgiese reseksie bied. Daarbenewens is *ACTG2* geïdentifiseer as die genetiese faktor in VSCM patologie; daarom kan die studie nuwe inligting verskaf oor die genetiese faktore van ADL.

Doel

Hierdie projek poog om die gene wat verband hou met die ontwikkeling van die enteriese senuweestelsel (*RET, NRG1, SOX10, EDNRB*) en gladdespierselle (*ACTG2*) te bestudeer wat bydra tot HSCR en ADL in die Suid-Afrikaanse neonaatbevolking.

Metodes

Sewentien volbloedmonsters is na die ingeligte toestemming van die HSCR-deelnemers afgehaal; waarvan slegs 14 monsters vir genotipering ingesluit is en 9 monsters is gekies vir RNA-analise gebaseer op die gehalte van onttrek DNA en RNA onderskeidelik. Vyf volledige bloedmonsters is ook by ADL-pasiënte ingesamel na ingeligte toestemming. RNA monsters van die HSCR kohort was omgekeerde transkribeerde en kwantitatiewe polimerase kettingreaksie uitgevoer. DNA monsters van HSCR en ADL monsters is gesif vir variante in

die *ACTG2* exons deur tweerigting Sanger volgorde. Nuwe variante is in siliko geanaliseer om hul patogeniteit te bepaal.

Resultate en bespreking

In beide HSCR- en ADL-kohorte is die variant K119E / R waargeneem in 64% (9/14) en 60% (3/5) van die studiepopulasie; K119E / R sal waarskynlik as 'n siekteveranderings funksie funksioneer, aangesien dit ook in die kontrolemonsters ses uit nege individue waargeneem word. Variante S345L en W357G in exon 10 met waarskynlike beduidende effek in die patogenese van ESM is slegs in die HSCR kohort geïdentifiseer. Die ADL-kohort het polimorfiese introniese variante voorspel om die eksoniese splytareas te verskuif, naamlik g> c -IVS12 exon 3 en c> t -IVS3 exon 5. Differensiële uitdrukking van ENS gene *EDNRB*, *RET*, *SOX10* en *NRG1* wat verband hou met ENS ontwikkeling in die HSCR kohort is nie bereik as gevolg van eksperimentele faktore nie.

Afsluiting

ACTG2 enkodeer 'n enteriese gladdespier γ-2 actien wat 'n sleutelrol speel in die kontraktiele proteïene van ESM. Die data dui daarop dat 'n spierkomponent bestaan in HSCR etiologie wat verder in vitro ondersoek behoort te word en verdere insigte in genetiese faktore wat kan bydra tot ADL patogenese.

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Table of Contents

Declaration.....i

Abstract.....ii

Opsomming.....iv

Acknowledgements.....vi

Table of Contents.....vii

List of Figures.....x

List of Tables.....xii

List of abbreviations.....xiii

Symbols.....xiv

Units of measure.....xv

CHAPTER 1.....1

1. Introduction.....1

1.1. Background.....1

1.2. Problem statement.....1

CHAPTER 2.....3

2. Literature Review.....3

2.1. Hirschsprung's disease.....3

2.1.1. *RET*.....4

2.1.1.1. The *RET* and *GDNF* pathway.....5

2.1.2. Other genes.....7

2.1.2.1. EDNRB.....8

2.1.2.2. SOX10.....9

2.1.2.3. NRG1.....9

2.1.3. Development of the enteric nervous system.....10

2.1.4. Disease Aetiology.....11

2.1.4.1. RET deactivation.....11

2.1.4.2. RET activation....12
2.1.5. Comorbidities....13
2.1.5.1. Down's syndrome....13
2.1.5.2. Type 4 Waardenburg syndrome....13
2.2. African Degenerative Leiomyopathy....13
2.2.1. Genetic aetiology of ADL....14
2.2.1.1. ACTG2....15
2.2.1.2. MYH11....15
2.2.2. Enteric smooth muscle development....15
2.3. Therapeutic measures....16
2.4. Aim....17
2.5. Objectives....17
CHAPTER 3....18
3. Materials and Methods....18
3.1. Patient recruitment....18
3.2. Gene selection....18
3.3. RNA extraction....18
3.4. DNA extraction....20
3.5. Reverse Transcription and cDNA synthesis....21
3.6. Real Time Quantitative Polymerase Chain Reaction....22
3.7. Polymerase Chain Reaction (PCR)....23
3.8. Agarose gel electrophoresis....24
3.9. Sequencing....24
3.10. *In Silico* analysis....25
3.10.1. Pathogenicity prediction....25
3.10.2. Secondary structure prediction....26
3.10.3. Human Splicing Finder....26

3.10.4. PyMOL....26
CHAPTER 4....27
4. Results....27
4.1. HSCR ENS genes....27
4.2. Smooth muscle....30
CHAPTER 5....40
5. Discussion....40
5.1.1. Gene expression analysis of HSCR....40
5.1.2. HSCR and *ACTG2* genetic factors....41
5.1.3. Genetic basis of ADL....42
5.1.4. Mutations in HSCR and ADL....44
5.2. Conclusion....44
5.3. Limitations....45
5.4. Future Study....45
CHAPTER 6....46
Article 1....46
Article 2....47
CHAPTER 7....50
Appendices....50
References....51

List of Figures

Figure 2.1: The structure of RET showing the different domains of RET including the splicing isoforms RET9 (black), RET43 (brown) and RET51 (blue)…….5

Figure 2.2: A schematic diagram of RET activation. The GDNF family ligands and their respective GPI anchored co-receptors on the lipid raft form a complex and each bind to RET and activate it……..6

Figure 2.3: Schematic diagram generated by Alves and colleagues demonstrating HSCR genes of interest *RET, EDNRB, SOX10* and *NRG1* interactions within the cell through their pathways…….8

Figure 2.4: Cross section of the intestine showing the organization of the PNS plexi and the musculature of the intestinal walls……10

Figure 4.1: Protein association network of the HSCR genes of interest generated with GENEMania, showing the genes co-expression and shared pathways……28

Figure 4.2: RT-qPCR amplification diagram of the HSCR cohort illustrating the gene expression of the HSCR cohort as number of cycles on the x-axis and the normalized reporter value on the y-axis as a log measure……..39

Figure 4.3: Amino acid chromatograph showing the S345L mutation occurring on the *ACTG2* exon 10 of one of the HSCR patients analysed on FinchTV……..30

Figure 4.4: Tertiary structure modelling of the *ACTG2* K119E variant and the neighbouring aa residue in close bonding distance; showing the variant Glu in cyan, Lys at position 114 and 117 in green and Trp at position 80 in magenta generated with PyMOL……..34

Figure 4.5: Protein structure prediction model of the *ACTG2* variant S345L generated with SWISS Model showing the wild type with a shorter side chain (**A**) and the mutant with an extended side chain (**B**) also showing the amino acids Ser and Leu localization on the protein……..…35

Figure 4.6: Amino acid chromatograph illustrating a heterozygous base exchange T>G on *ACTG2* exon 10 producing the W357G variant analysed on FinchTV…….36

Figure 4.7: Protein modelling of the *ACTG2* variant W357G showing the wild type with an aromatic side chain Trp (**A**) and the mutant with a short Gly side chain (**B**) generated with SWISS Model……...36

Figure 4.8: Modelled secondary structure of ACTG2 protein generated with PyMOL showing the wild type aa residue W on position 357 in red and neighbouring aa residues F353 in yellow and Y134 in cyan within bonding distance of each other……..37

Figure 4.9: Exonic splice silencer g>c -IVS12 on the intronic region prior to exon3 of *ACTG2*; showing a homozygous variant with G and C bases at the same position analysed on FinchTV……...38

Figure 7.1: Mutiple sequence alignement of the ACTG2 protein of the species: human, mouse, horse, loxaf (Africn elephant) and myolu (little brown bat) generated with CLUSTALW showing the aligment score between the species and a rooted phylogenic tree showing the species conservation of the protein……..51

List of Tables

Table 3.1: Patient demographics of the HSCR and ADL cohort……..18

Table 3.2: RT-qPCR components and volume of each component used for the real time amplification of the HSCR cohort samples……..21

Table 3.3: *ACTG2* exons primers designed on the Oligo Analyzer platform, showing the optimised annealing temperature of each exon primer set……22

Table 3.4: Components used in the PCR amplification of the ACTG2 exons for both HSCR and ADL cohorts……...23

Table 4.1: HSCR samples showing the RNA concentration quantified with the NanoDrop and Bioanalyzer machines and their respective RIN value……..26

Table 4.2: Known and novel ACTG2 variants observed in the HSCR cohort……..30

Table 4.3: ACTG2 screening of the healthy control samples observed to have the intronic C>T -IVS6 and K119E/R variants……31

Table 4.4: *ACTG2* single nucleotide polymorphisms (SNPs) identified in HSCR patients showing the pathogenicity score as predicted by the nsSNP predictor tools, the number of patients affected and the exon which each variant occurs…….32

Table 4.5: *ACTG2* variants observed in the ADL cohort, showing the observed variants and the exons they occur in……..36

Table 4.6: *ACTG2* SNPs identified in the ADL cohort, showing the pathogenesis prediction scores of each variant, the exon of occurrence and the number of patients affected…….39

List of abbreviations

ΔRn	Normalized Reporter value
aa	Amino Acid
ADL	African Degenerative Leiomyopathy
ACTG2	Actin gamma 2
Buffer EL	Erythrocyte lysis buffer
C	Cysteine
CADD	Combined Annotation Dependent Depletion
cDNA	Complementary DNA
Ct	Cycle threshold
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphospate
E	Glutamic acid
EDNRB	Endothelin Receptor B
ENS	Enteric Nervous System
G	Glycine
HSF	Human Splicing Finder
HSCR	Hirschsprung's disease
K	Lysine
L	Leucine
mRNA	Messenger RNA
Mut	Mutant
MYH11	Myosin Heavy chain 11
NRG1	Neuregulin 1
nsSNP	non-synonymous SNP
PCR	Polymerase Chain Reaction

P	Proline
R	Arginine
rs	Accession number
RET	REarranged during Transfection
RIN	RNA Integrity Number
RNA	Ribonucleic Acid
RT-qPCR	Real Time Quantitative PCR
S	Serine
SIFT	Sorting Intolerant From Tolerant
SMC	Smooth Muscle Cells
SNP	Single Nucleotide Polymorphism
SOPMA	Self-Optimized Prediction from Multiple Alignment
SOX10	Sry Box 10
V	Valine
VSCM	Visceral myopathy
W	Tryptophan
WT	Wild type
Y	Tyrosine

Symbols

%	Percentage
^{o}C	Degree Celsius
α	Alpha
β	Beta
γ	Gamma
π	Pi

Units of measure

M	Molar
mg/ml	Milligram per millilitre
ml	Millilitre
ng/µl	Nanogram per microliter
µl	Microliter
rpm	Revolutions per minute
V	Volt

CHAPTER 1

1. Introduction

1.1. Background

Gut motility is a well-co-ordinated movement facilitated by a combination of enteric neurons, smooth muscle cells and interstitial cells of Cajal. These components function co-ordinately to assist in the transportation and absorption of nutrients, and the elimination of waste from the gastrointestinal tract (Burzynski et al., 2009; Schlieve et al., 2017). Disorders affecting gastrointestinal motility such as Hirschsprung's disease and African degenerative leiomyopathy are part of gastrointestinal motility deficit spectrum, resulting from either neuropathic or myopathy deficit respectively. These debilitating diseases are prevalent in young children and have thus far proven to be problematic for both paediatricians and gastroenterologists alike; resulting from their ability to progress to a severe condition such as enterocolitis post successful resection surgery (Levitt et al., 2010).

Affected patients rely on corrective surgery to alleviate the effects of the disorders; however, for some individuals the quality of life is reduced, and they may require repeated surgery throughout their life due to disease persistence (Friedmacher and Puri, 2011; Kessmann, 2006). The genetic basis of HSCR has been elucidated through research, which has assisted parents by genetic counselling. On the other hand, the persistence of dysmotility in some patients has led to the investigation of other environmental and genetic risk factors contributing to disease recurrence post resection surgery. The genetic insights into these gastrointestinal diseases tested in this study may provide a model for the development of novel treatment and disease management.

1.2. Problem statement

A small number of HSCR patients are affected by recurrent motility defects following successful resection surgery, which further predispose to enterocolitis. Although the enteric nervous system genes namely (*RET, GDNF, GFRα, EDNRB, SOX10, PSP* etc.) have been extensively studied in HSCR cases; however, they do not provide sufficient information on the

possible cause of the morbidity persistence. Therefore, studying genes responsible for smooth muscle development such as *ACTG2*, which are the attachment area where ENS pass their current for contraction may provide information essential towards understanding the risk of recurrence.

Further study of these smooth muscle gene (*ACTG2*) may also provide information regarding the molecular basis of African degenerative leiomyopathy; which is generally accepted as an acquired disease. In addition, cases of familial occurrences have been reported suggesting a possible genetic etiopathogenesis, hence comparing the smooth muscle gene variation between these gastro-motility diseases may provide vital information regarding the affected genes.

Keywords: Hirschsprung's disease, Enteric nervous system, Enteric smooth muscle, African degenerative leiomyopathy

CHAPTER 2

2. Literature Review

2.1. Hirschsprung's disease

Hirschsprung's disease (HSCR) (OMIM 142623) has been labelled as one of the most understudied genetic disorders in the African population; although it has been reported to occur in 1:5000 live births in the Caucasian population (Bahrami et al., 2018). Recent studies have shown that only 20-40% of neonates affected by HSCR in the African population have been diagnosed with the disorder compared to 90% diagnosis in European countries (Mabula et al., 2014). HSCR is a congenital malformation of the distal gastrointestinal tract, resulting from the absence of intrinsic ganglion cells in the wall of the hind gut of affected patients (Eketjäll and Ibáñez, 2002; Iwashita et al., 1996). It is characterized by the functional obstruction of the bowel and failure to pass stool within the first 24 hours of life. This may be accompanied by severe constipation, colonic distention in neonates and enterocolitis in adults (Basel-Vanagaite et al., 2007; Garcia-Barceló et al., 2004; Mandhan, 2011).

HSCR does not follow the Mendelian mode of inheritance and is a sex biased congenital anomaly with a male: female ratio of 4:1 (Amiel et al., 2008). It is commonly classified into two types depending on the extent of the aganglionosis of the affected colon, namely: short segment (S-HSCR) and long segment (L-HSCR). S-HSCR affects the recto-sigmoid region of the bowel, meanwhile L-HSCR extend beyond the recto-sigmoid region thus affecting the entire colon. Additionally, S-HSCR is the most common type of congenital aganglionosis observed in 80% of reported HSCR cases. Other forms of HSCR have been reported where the aganglionosis extend beyond the colon and affect the small intestine although these are rare. Total colon aganglionosis (TCA) occurs as a result of lack of ganglion cells on portions of the small intestine in addition to the colon (Alves et al., 2013). Rarely the aganglionosis affects both the entire colon and small intestine, this is classified as total intestine aganglionosis (TIA) (Amiel and Lyonnet, 2001).

HSCR pathogenesis has been shown to be a consequence of the disruption of the normal development of the enteric nervous system (ENS). Therefore, its pathogenesis can be understood conceptually through the study of the cellular and molecular mechanisms of genes

associated with normal ENS development. The REarranged during Transfection (RET) proto-oncogene is the major controlling signalling pathway of ENS development; with at least one *RET* allele alteration identified in individuals affected by HSCR (Amiel and Lyonnet, 2001; Gui et al., 2017; Julies et al., 2001). *RET* coding mutations have been reported in both familial and sporadic HSCR cases occurring at 50 and 15-35% respectively. Serra and colleagues 2009, reported that mutations also occur on the enhancer sequence on intron 1 which has a stronger predisposition to HSCR (Serra et al., 2009). The intronic alterations present in the form of two single nucleotide polymorphisms (SNPs) rs2506004 (SNP1) and rs2435357 (SNP2); the latter is known to disrupt the binding site of transcription factor *SOX10* subsequently affecting the expression of *RET* (Kapoor et al., 2015; Moore and Zaahl, 2012; Núñez-Torres et al., 2011).

2.1.1. *RET*

REarranged during Transfection *(RET)*, which encodes a receptor tyrosine kinase *(*RTK*)* maps to chromosome 10q11.2 (Taccaliti et al., 2011) comprises of 21 exons. RET is a 1114 transmembrane amino acid; its structure consists of the extracellular cadherin-like and intracellular tyrosine domains and a cysteine rich region. These components are all essential for RET phosphorylation and downstream signalling (Amiel and Lyonnet, 2001; Wagner et al., 2012) (figure 2.1). *RET* binds growth factor receptor of the glial cell line derived neurotrophic factor (GDNF) on neural crest cells (Ibáñez, 2013; Santoro et al., 2004); which functions to promote ENS development.

The alternative splicing of RET generates its three isoforms namely the long, intermediate and short RET isoforms; which differ variably by amino acid length at the C-terminus. These isoforms are commonly referred to as RET51, RET43 and RET9 (Arighi et al., 2005) (figure2.1). RET9 and RET51 are the two main isoforms, they share tyrosine (Y) residues except the Y1096 which is specific to RET51 (Ibáñez, 2013; Little, 2015); the phosphorylation of the Y residues trigger the activation of RET signalling pathways.

RET is reported to be responsible for triggering three signalling pathways; the mitogen activated protein kinase (MAPK), the phospholipase C- gamma (PLCγ) and the phosphatidylinositol-3 kinase (PI3K) pathways (Ibáñez, 2013; Lundgren et al., 2012). The roles of the MAPK and PI3K pathways in the nervous system involves the promotion of neurite outgrowth and survival of neurons (Sariola and Saarma, 2003). The PLCγ pathway plays a

crucial role in regulation of the intracellular calcium ion levels (Lundgren et al., 2012; McCain, 2013; Sariola and Saarma, 2003).

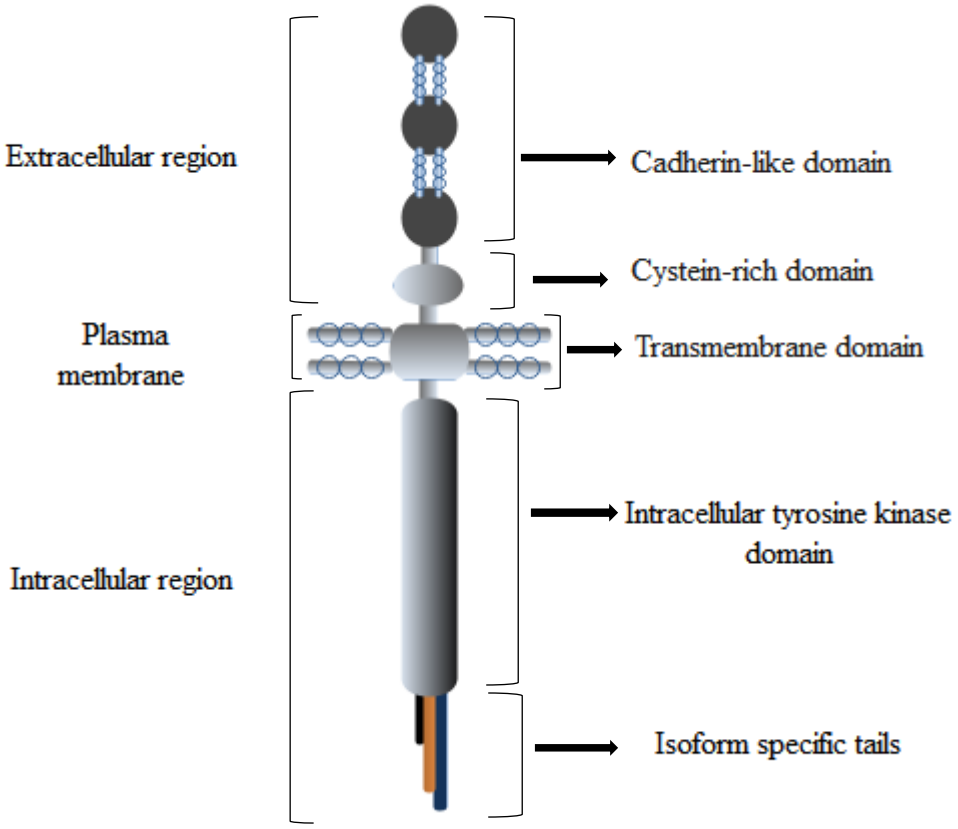

Figure 2.1: The structure of RET showing the different domains of RET including the splicing isoforms RET9 (black), RET43 (brown) and RET51 (blue). Adapted from (Phay and Shah, 2010) Copy Right license 4474300195937 (Clinical Cancer Research)

2.1.1.1. The *RET* and *GDNF* pathway

Under normal cell growth, RET is exclusively activated by the binding of a soluble ligand complex; composed of the glial cell line derived neurotrophic factors (GDNF) family ligands and the co-receptor of the GDNF family receptor α (GFRα) (Anderson et al., 2013). The GDNF ligand family include; glial cell line derived neurotrophic factor (GDNF), artemin (ARTN), neurturin (NTRN) and persephin (PSPN) (Mason, 2000; Wagner et al., 2012). GDNF receptors require gylcosylphosphatidylinositol (GPI) anchored co-receptors to interact with RET on the cell surface, commonly known as GDNF family receptor α (GFRα 1-4) (figure 2.2).

Activation of RET is essential for the survival of pre-enteric neural crest cells in the foregut mesenchyme (Natarajan et al., 2002), resulting in mature enteric neural crest cells and also provides a proliferative signal to the enteric neural crest cells. GDNF, NTRN, ARTN and PSPN ligands bind GPI anchored receptors GFRα1, GFRα2, GFRα3 and GFRα4 respectively; in order to signal through RET (figure 2.2) (Wagner et al., 2012). Multiple downstream pathways are stimulated by the activation of *RET*, resulting in the promotion of cell growth, survival, proliferation and differentiation.

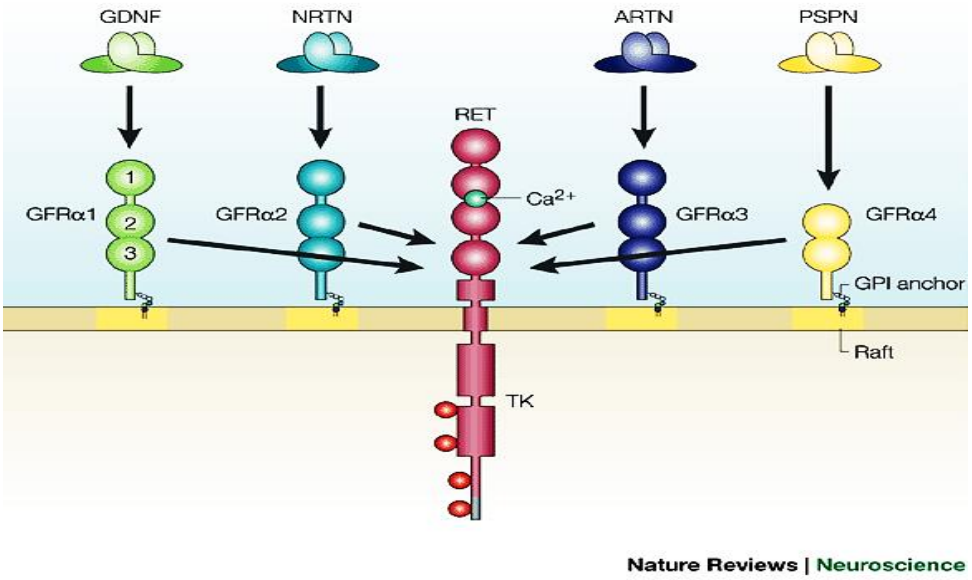

Figure 2.2: A schematic diagram of RET activation. The GDNF family ligands and their respective GPI anchored co-receptors on the lipid raft form a complex and each bind to RET and activate it. Taken from (Airaksinen and Saarma, 2002). Copy Right licence 4446391044778 (Elsevier and Copyright Clearance Center). TK= Tyrosine Kinase

One of RET ligands, GDNF known to be the main ligand in RET activation, its gene maps to chromosomes 5p12-13.1. GDNF has been reported to be essential for the survival of neuronal cells from both the peripheral nervous (PNS) and central nervous systems (CNS) (Taraviras et al., 1999). During ENS development, GDNF has been reported to be involved in directing the enteric neural crest cells (ENCC) during their caudal migration to the entire gut (Holschneider and Puri, 2007); while acting as a chemoattractant to the ENCC. Additionally, GDNF functions to give proliferative signals to the ENS progenitors in the colon (Mundt and Bates, 2010), and

also plays a pivotal role in preventing NCCs from early differentiation into neurons (Anderson et al., 2013) supporting its role in gene regulation.

Although studies have shown that mutations occurring solely in the *GDNF* rarely contribute to reduced RET activation (Borghini et al., 2002); a characteristic known to predispose to the aganglionosis of the distal bowel. Numerous studied have demonstrated that *GDNF* mutations which result in HSCR phenotype are a consequence of a combination of mutations in both *GDNF* and the susceptibility locus *RET*.

2.1.2. Other genes

Hirschsprung's disease has been classified as a multigene disease with the *RET* proto-oncogene described as the major susceptibility gene; however other genes also play a role in its pathogenesis which account for at least 7% to the disease susceptibility. Fourteen HSCR susceptibility genes in addition to the *RET* proto-oncogene have been identified. These genes belong to three categories: (i) genes involved in the activation of the RET pathway (*RET, GDNF, GFRα, L1CAM, NTN* and *PSP*), (ii) genes implicated in the EDNRB pathway (*EDNRB, EDN3, ECE-1, NRG1* and *NRG3*) and (iii) RET/EDNRB pathway transcription factors *PHOX2B, ZFXH1B, PAX3* and *SOX10.*

The downregulation of these genes negatively affect their role in neurite migration, localization, proliferation and differentiation; which are essential for ENS development during embryogenesis. Alves and colleagues 2013, conducted research which outlined the correlation between *RET, SOX10, EDNRB* and *NRG1* within cells during embryogenesis (figure 2.3), which this study has selected to analyse further in real time indicated by red circles on figure 2.3 (Alves et al., 2013).

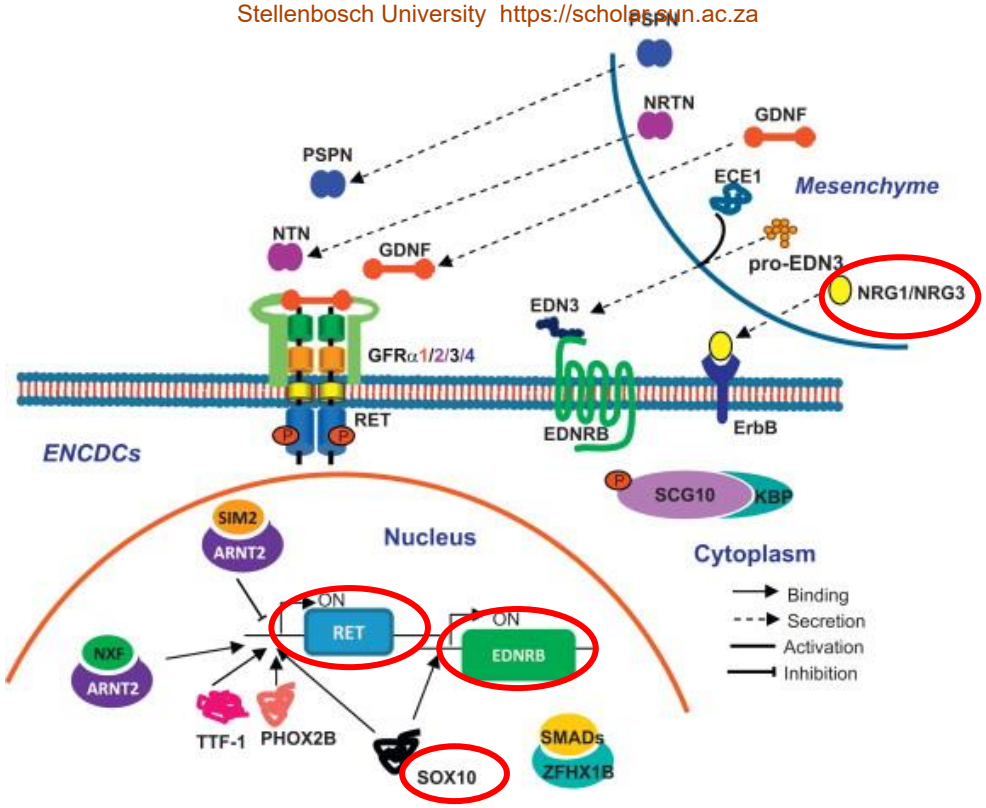

Figure 2.3: Schematic diagram generated by Alves and colleagues demonstrating HSCR genes of interest *RET, EDNRB, SOX10* and *NRG1* interactions within the cell through their pathways. Taken from (Alves et al., 2013) Copy Right licence 4459281032094 (Elsevier and Copyright Clearance Center). ENCDC=enteric neural crest-derived cells

2.1.2.1. EDNRB

The endothelin pathway is the second most common pathway linked to HSCR development and its phenotype (Sánchez-mejías et al., 2010). It includes genes such as endothelin cleaving enzyme 1 (*ECE1),* endothelin 3 (*EDN3)* and endothelin receptor type-B *(EDNRB)* accounting for at least 3-7% of reported HSCR mutations (Barlow et al., 2003). *EDNRB* maps to chromosome 13q22 that is activated by the binding of mesenchymally-synthesized EDN3. EDN3 is the result of enzymatic cleavage of the inactive intermediate EDN by ECE1 (Saldana-Caboverde and Kos, 2010).

Research has demonstrated that EDNRB is responsible for the formation of embryonic cells with different fates post embryonic development, such as enteric cells and produces melanocytes (Barlow et al., 2003; Goldstein et al., 2013; Mccallion and Chakravarti, 2001; Saldana-Caboverde and Kos, 2010); which are precursor cells that produce the pigment melanin. In addition, EDNRB is also involved in preventing the early differentiation of the neural crest precursor cells through timed regulation that maintains them in a proliferative state

during their migration to the distal portion of the digestive tract (Goldstein et al., 2013; Nagy and Goldstein, 2006).

2.1.2.2. SOX10

The multipotent sex determining region Y box 10 (SOX10) forms part of a large group of transcription factors; comprising 466 amino acids. *SOX10* forms part of a family of genes with a highly conserved mobility group DNA binding domain in addition to a C-terminal domain (Sánchez-Mejías et al., 2010). It is essential for the development of both melanocyte and neural crest cells (Han et al., 2018). SOX10 function in the ENS development include neurite proliferation, migration and differentiation (Holschneider and Puri, 2007).

SOX10 is also involved in the transcriptional regulation of both *RET* and *EDNRB* (Lake and Heuckeroth, 2013). As a consequence of its role in regulation of both genes; it has been reported to be an important ENS transcription factor. Sánchez-Mejías and colleagues 2010, reported that current research has not identified isolated *SOX10* mutations responsible for HSCR morbidity (Sánchez-Mejías et al., 2010; Sham et al., 2001). However, *SOX10* mutations are associated with syndromic type 4 Waardenburg syndrome-HSCR known to have a high HSCR penetrance.

2.1.2.3. NRG1

A member of the neuregulin family, neuregulin1 *(NRG1*) is an important ENS maintenance gene specifically involved in the formation of synapses, differentiation of nerve cells and the outgrowth of neurites (Barrenschee et al., 2015). NRG1 functions as a signalling glycoprotein which forms a heterodimeric complex with ErbB2/ErbB3 receptor tyrosine kinases (Kapoor et al., 2015). Similar to *RET* intronic SNP1 and SNP2, *NRG1* has risk variants on intron 1 known to have a higher predisposition effect to HSCR namely rs16879552 and rs7835688 (Gui et al., 2017; Jiang et al., 2017; Tang et al., 2011). Additionally, *NRG1* coding mutations have been observed in at least 6% of HSCR cases (Alves et al., 2013). It has been further reported that HSCR predisposition is increased by the concurrence of both *RET* and *NRG1* intronic SNPs (Gui et al., 2017).

2.1.3. Development of the enteric nervous system

ENSis a component of the PNS made up of neurons and glia that form an interconnected network of enteric ganglia comprised of an outer and inner plexi; commonly known as myenteric and submucosal plexi respectively (Barlow et al., 2008) (figure 2.4). The plexi are located within the gut wall muscle layers; the myenteric plexus is located between the circular and longitudinal portion of the muscle layers and can be found throughout the digestive tract (Mills and Stappenbeck, 2013). Meanwhile the submucosal plexus is exclusively located in the small and large intestine (Goldstein et al., 2013; Heanue and Pachnis, 2007) under the submucosal layer (Figure 2.4). The ENS functions to co-ordinately regulate intestinal motility (transportation of water and electrolytes), secretion, nutrient absorption and blood flow through the activation of its intrinsic reflexes (Goldstein et al., 2013). Subsequently contributing to the maintenance of the lumen and gut wall by microbiota composition constraining; a role reported to assist in maintaining commensal microbial communities (Rolig et al., 2017), thereby promoting a healthy gut.

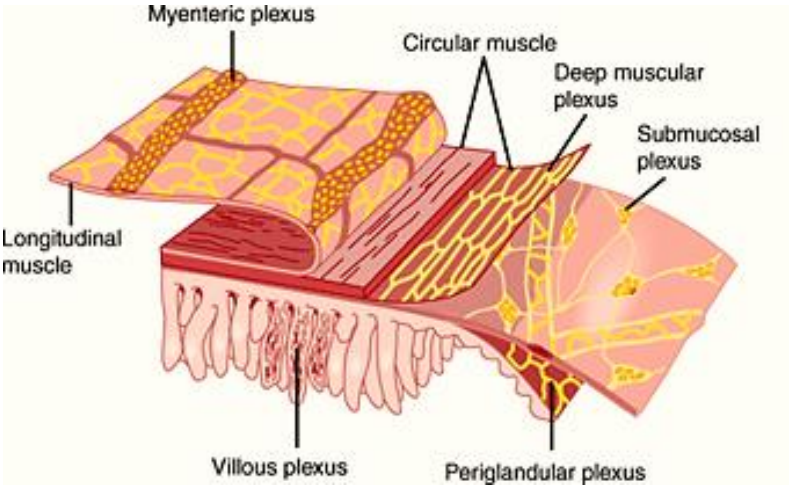

Figure 2.4: Cross section of the intestine showing the organization of the PNS plexi and the musculature of the intestinal walls. Adapted from (Furness et al., 2014). Copy Right license 4467570350306 (Copyright Clearance Springer eBook)

During embryogenesis neural crest-derived (NC) cells invade the bowel and migrate recto-caudally into the colon; the migration is coupled with extensive proliferation and differentiation of NC into neurons and glia (Lake and Heuckeroth, 2013; Nagy and Goldstein, 2006). The neurons and glia subsequently condense into ganglia forming a network of active neurons and glia in the distal bowel. During week 7 of gestation the vagal portion of the neural tube gives

rise to vagal ENS progenitors known as pre-enteric neural crest cells (pENCC); which subsequently invade the mesenchyme (Vandewalle et al., 2005; Wallace and Burns, 2005).

The invasion of the pENCCs into the mesenchyme is facilitated by the expression of transcription factor PHOX2B and the receptor tyrosine kinase RET (Burzynski et al., 2009; Laranjeira and Pachnis, 2009). The pENCCs migrate dorsally thereby inducing the expression of PHOX2B and RET; once in the mesenchyme, the cells are therefore known as enteric neural crest cells (ENCC). RET and SOX10 initiate the migration of the cells recto-caudally; where SOX10 also functions as ENCC marker while maintaining the cells in progenitor state (Butler Tjaden and Trainor, 2013). The progenitor arrest of ENCC enables the colonization of the entire gastrointestinal tract ultimately giving rise to the ENS.

Due to this well coordinated migratory and differentiation, *RET* and the other developmental genes are essential to the development of the ENS. In addition to migration, RET signalling during embryogenesis supports the survival, neuronal differentiation, proliferation and colonization of ENS precursors and neurite growth. The successful colonization of NC in the bowel result in the ability to control peristaltic and secretory activity of the gut wall (Natarajan et al., 2002). Failure of NC to effectively colonize the bowel has clinical implications that lead to the aganglionosis of the ENS; consequently, causing diseases that affect gastrointestinal motility such as Hirschsprung's disease.

2.1.4. Disease Aetiology

2.1.4.1. RET deactivation

Loss of function or the inactivation of RET leads to the aganglionosis of the ENCC; subsequently causing the aganglionosis of the distal portion of the intestine which clinically manifest as HSCR. It has been reported that both germline point mutations (deletion, insertion and substitution) and non-coding mutations occurring on *RET* lead to a partial loss of function phenotype (Jannot et al., 2013; Myers et al., 1999). These mutations confer a cell type specific decrease in functional protein on the cell surface (Wagner et al., 2012); resulting in the inactivation of RET.

RET inactivation which results in HSCR disease occurs as a consequence of at least one of four mechanisms (Kurokawa et al., 2003; Takahashi, 2001):

1. Incorrect folding of RET subsequently leading to the impairment of RET cell surface expression.
2. Mutation in the kinase domain of RET, that leads to the termination of the RET kinase activity.
3. Impairment of the binding adaptor proteins as a result of mutations in the carboxy-terminal tail.
4. Mutations in the kinase domain of RET which reduce the activation of PLCγ pathway.

As a functional consequence of RET inactivation, migration of neural crest cell during embryonic development is disrupted leading to congenital aganglionosis of the colon (Liang et al, 2014). ENCCs undergo apoptotic cell death resulting in the elimination of ENS precursors from the gastrointestinal tract (Liang et al., 2014). RET inactivation occurs in the absence of the GDNF-GFRα ligand complexes responsible for activating RET in addition to a variety of frame-shift, missense and nonsense mutations observed in the coding sequence (Kurokawa et al., 2003).

2.1.4.2. RET activation

In contrast, gain of function mutation which result from ligand-independent constitutive activation of RET is responsible for development of multiple endocrine neoplasia (MEN) type 2 A and B (MEN2A and MEN2B) cancers (Santoro et al., 2004; Wagner et al., 2012). MEN displays an autosomal dominant mode of inheritance (Lundgren et al., 2012); characterized by medullary thyroid carcinoma (MTC) including familial MTC (FMTC). Wild type activation of RET occurs in the presence of a multi-protein ligand complex comprising of GDNF ligands and their co-receptors GFR-α (Takahashi, 2001).

Mutations responsible for MEN and MTC cancers occur in the cysteine rich (C-rich) portion of RET (Arighi et al., 2004). In this C-rich region a substitution of one of the highly conserved RET cysteine residues results in an unpaired cysteine; as a consequence this molecular modification enables the constitutive activation of RET (Arighi et al., 2005; Santoro and Carlomagno, 2013). These mutations are classified into two groups based on the part of the RET proto-oncogene they affect; those affecting the tyrosine kinase domain accounting for MEN2A and FMTC pathogenesis and those affecting the extracellular domain which are associated with MEN2B pathogenesis (Arighi et al., 2005; Wagner et al., 2012).

2.1.5. Comorbidities

2.1.5.1. Down's syndrome

Frequently HSCR occurs with other genetic disorders; the common examples are Down's syndrome (DS), Mowat-Wilson Syndrome, and Waardenburg syndrome (WS) in the affected patients. DS is the most common genetic disorder associated with HSCR; Heuckeroth 2015, reported that at least 2-10% of neonates with DS also harbour HSCR mutations (Heuckeroth, 2015). Furthermore, research suggests that mutations in chromosome 21 increase the affected patients' susceptibility to HSCR (Jannot et al., 2013). DS contributes to HSCR aetiology with an estimated 40% risk factor (Friedmacher and Puri, 2013; Jannot et al., 2013). Themajority of the *RET* mutations observed in patients affected with a combination of DS-HSCR occur in the non-coding polymorphism of the *RET* proto-oncogene enhancer element on intron 1 which affects the *SOX10* binding site.

2.1.5.2. Type 4 Waardenburg syndrome

Mutations in *EDNRB* have been reported to play a role in the development of the type 4 Waardenburg syndrome (WS4) in addition to aganglionosis of the colon, also referred to as Shah-Waardenburg syndrome. WS4 is a neurocristopathy characterized by sensorineural hearing and hair loss, skin and iris hypopigmentation in addition to HSCR. Nonetheless, the prevalence of WS associated with HSCR is rare; only occurring in only 4 in 1 million of reported cases (Karaca et al., 2009; Mahmoudi et al., 2013). The pathogenesis of WS4 involves other ENS genes such as *SOX10* and the EDNRB ligand *EDN3* (Amiel and Lyonnet, 2001), although the mechanism of action is not fully understood.

2.2. African Degenerative Leiomyopathy

Chronic intestinal pseudo-obstruction syndromes (CIPOs) include a variety of rare disorders affecting the motility of the gastrointestinal tract and peristalsis. This group of disorders such as hollow visceral myopathy (HVM) and megacystis microcolon intestinal hypoperistalsis syndrome (MMIHS) affect different parts of gut wall muscles (Matera et al., 2016; Moore et al., 2002); the former is associated with the pathogenesis of the distal gut. African degenerative

leiomyopathy (ADL) is a distinct form of visceral myopathy (VSCM) also known as Bantu pseudo-Hirschsprung's disease (OMIM 155310) (Rode et al., 1992). ADL is prevalent in the Black African population; mainly those residing in the Southern, East and Central Africa.

ADL is an enteric smooth muscle dysmotility disorder characterized by the impairment of the gastrointestinal propulsion without myenteric neuronal pathogenesis (Lehtonen et al., 2012). It consequently results in abdominal pain and enlargement, impaired nutrient absorption which leads to malnutrition, distention and sometimes leads to death (Klar et al., 2015; Rensburg et al., 2012). Chitnis and colleagues further reported that ADL progressively affects the gastrointestinal and genitourinary systems (Chitnis et al., 2011). In addition, ADL occasionally display the inherent ability to progress proximally from the distal bowel into the small intestine; resulting in some patient relying on total parenteral nutrition.

2.2.1. Genetic aetiology of ADL

Previously, it was generally accepted that ADL was an acquired myopathic disorder with varying onset from birth to later in childhood (Halim et al., 2016). Its aetiology was linked only to environmental toxins coupled with poor nutrition; known to progressively degenerate the distal gut smooth muscle walls (Moore et al., 2002). Van Rensburg and colleagues 2012, prospectively studied *RET* variants in a rare familial case of ADL with the aim of providing insight regarding a molecular basis of the disease (Van Rensburg et al., 2012); from which numerous *RET* variants were identified.

ADL often occurs sporadically, displaying an autosomal dominant pattern (Klar et al., 2015) whereas in the rare familial cases it displays heterozygosity with autosomal recessive patterning (Moreno et al., 2016). Although its genetic aetiology has not yet been elucidated; studying the actin and/ or myosin genes which have been associated with mutations in VSCM may aid in furthering the study towards the molecular basis of ADL. Mutations associated with smooth muscle actin and myosin in VSCM patients have been extensively studied under developing research.

2.2.1.1. ACTG2

Actins are highly conserved family of proteins which form part of the cytoskeleton and exists in three isoforms, namely alpha (α), beta (β) and gamma (γ); each isoform has an essential function in gut motility. The β and γ isoforms especially play a role in the mediation of cell motility and form part of the cytoskeleton maintenance components (Sonnemann et al., 2006). Actin-γ-2 (*ACTG2*) is a smooth muscle gene encoding the γ-actin isoform, commonly known to be exclusively expressed in the urogenital and intestinal tracts of the enteric smooth muscle cells (Halim et al., 2016; Milunsky et al., 2017a). Meanwhile the α- and β- isoforms are found throughout the eukaryote cells as part of the cytoskeleton.

ACTG2 on chromosomal position 2p13.1 is essential for distal gut motility which facilitate the accurate contractile motion of the smooth muscle cells responsible in aiding nutrient absorption and digestion (Matera et al., 2016). It consists of 10 exons of which 8 (exons 3-10) undergo translation, mutations that occur in *ACTG2* exons have been associated with various enteric muscular diseases such as MMIHS, VSCM and CIPO (Halim et al., 2016; Ravenscroft et al., 2018; Wangler et al., 2014).

2.2.1.2. MYH11

Intestinal walls are lined by thick filaments known as myosin which are organized in a hexametric orientation in the lumen (Huang et al., 2018). Myosins are organized within the longitudinal and circular muscles as shown in figure 2.4; myosin proteins form part of the smooth muscle's major contractile and cell movement mechanisms. The gene responsible for encoding the myosin protein is known as myosin heavy chain 11 (*MYH11*) with a 16p13.11 chromosomal location (Kuang et al., 2011). *MYH11* is known as one of the single genes that encodes four transcripts resulting in the SM1, SM2 isoforms at the carboxyl terminus and SMA, SMB isoforms at the amino terminus (Babu et al., 2000); this occurs through alternative splicing. The structure of MYH11 is composed of the head region which interacts with actin and a tail region which interacts with other myosin tails.

2.2.2. Enteric smooth muscle development

Enteric muscle cell fibres are primarily composed of both actin and myosin filaments; with actin forming the thin filaments whereas the thick filaments make up the myosin (Kwartler et

al., 2014). In humans, the differentiation of the enteric smooth muscle cells (SMCs) during intestinal organogenesis is a process known to occur simultaneously with ENCC colonization (Graham et al., 2017). Although the SMC is formed independently from the neuronal network; it's contractile mechanism has been reported to be regulated by the ENS and interstitial cells of Cajal (ICC) (Bourret et al., 2017; Le Guen et al., 2015; Sanders et al., 2012). ENS provide sufficient current for the generation of contractile action of the smooth muscles. SMC plays an essential role in the gastrointestinal tract as the last effector of contraction in the hindgut and excretion of waste from the stomach (Goldstein et al., 2016). Consequently, the impairment of SMC leads to diseases of hindgut hypomotility without aganglionosis. Furthermore, the outer longitudinal muscle layer (figure 2.4) is the portion of the intestinal wall acutely affected by enteric smooth muscle degeneration (Lehtonen et al., 2012).

SMC is formed from the mesenchyme which arises from the splanchnic mesoderm; these undifferentiated cells elongate, cluster and migrate rostro-caudally during organogenesis (Bourret et al., 2017; Wallace and Burns, 2005). They express α-smooth muscle as an initial marker of cell differentiation; a process subsequently followed by the expression of γ-smooth muscle actin and smooth muscle protein 22 (Bourret et al., 2017; Faure et al., 2015; Graham et al., 2017). The expression of γ-smooth muscle actin and smooth muscle protein 22 signals that the cells have entered a determined phase. These determined cells enter a phase of differentiation mainly characterized by the expression of smooth muscle contractile proteins namely Calponin and Caldesmon (McKey et al., 2016). The expression of the contractile proteins marks the completion of the SM development during organogenesis.

2.3. Therapeutic measures

The current disease management available for HSCR involves corrective surgery employing the endorectal pull-through techniques (Friedmacher and Puri, 2011). These techniques are carried out by the removal of the aganglionic bowel segment and restoring functionality by joining the remaining normal bowel to the rectum. The commonly used operating techniques such as Duhamel's, Swenson's and Soave's pull-through operations are regarded as an advancement from the usually invasive and time consuming rectosigmoidectomy and rectal myotomy (Kasai et al., 1971). The outdated techniques were often preceded by a preliminary colostomy or ileostomy months prior the operation (Somme and Langer, 2004). In contrast, the current Duhamel's, Swenson's and Soave's pull-through techniques have improvements such as short hospitalization, one-step procedure and reduced stoma morbidity (De La Torre

and Langer, 2010; Georgeson and Robertson, 2004; Levitt et al., 2010). The improved techniques were first described in the 1980s with minimal use of laparoscopy (De La Torre and Langer, 2010).

Similarly, ADL requires abdominal surgery that may include the following procedures; laparoscopy assisted, ileostomy or gastrostomy procedures (Milunsky et al., 2017b) to alleviate the effects of the motility deficit; however, these do not always restore normal bowel function. Although surgical resection of the distal tract is currently the accepted method used for both HSCR and ADL remediation; some patients may experience residual aganglionosis post-surgery (Khong and Malcomson, 2015). Kessmann and colleagues 200, reported that 10-34% of HSCR patients whom have undergone successful resection surgery still present with persistent gastrointestinal defects throughout life; leading to enterocolitis or colonic rupture (Kessmann, 2006; Khong and Malcomson, 2015). As a result of such complications; close monitoring of patients post-surgery is recommended.

We hypothesize that the persistence of the dysganglionosis may be a result of both ENS and SMC pathogenesis contributing to the HSCR phenotype. In this study we analyse the association between SMC genes and recurrent HSCR by comparing the variants *ACTG2* between HSCR and ADL patients. Further studying the expression of the genes associated with ENS development.

2.4. Aim

This project aims to study the genes associated with the development of enteric nervous system (*RET, NRG1, SOX10, EDNRB*) and smooth muscle cells (*ACTG2*) that contribute to HSCR and ADL in the South African neonate population.

2.5. Objectives

1. Analyse the differential expression of *RET, NRG1, SOX10, EDNRB* in HSCR cohort.
2. Screening of *ACTG2* in HSCR sample set.
3. Prospectively analyse ADL sample set for variants in *ACTG2.*
4. Comparing *ACTG2* variants between HSCR and ADL patients.

CHAPTER 3

3. Materials and Methods

3.1. Patient recruitment

Ethical approval for the research project was granted by the Stellenbosch University Human Research Ethical Review Committee; ethical reference C2001/019. For the HSCR cohort, whole blood samples were collected pre-surgery with informed consent from 17 participants with histologically confirmed HSCR and nine non-HSCR controls at the Tygerberg Children's Hospital. For the ADL cohort whole blood samples were collected from five histologically confirmed ADL patients; three participants from Pietermaritzburg Medi-clinic and two participants from Red-Cross Children's Hospital patient demographics are outlined in table 3.1. Non-HSCR control samples were not age matched; for minor participants the informed consent was given by a parent or legal guardian. Additionally, 8 negative control samples for Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) were obtained with informed consent from the TB Meningitis study; ethical reference N16/11/142. The HSCR samples were used for both RNA and DNA analysis.

Table 3.1: Patient demographics of the HSCR and ADL cohort

Disease	Age range	Gender
HSCR	2weeks-5years	14 males, 3 females
ADL	10-24years	4 males, 1 female

3.2. Gene selection

The genes used for the RT-qPCR study were selected based on their protein-protein interaction based networks using GENEMania interaction database https://genemania.org/ .

3.3. RNA extraction

Ribonucleic Acid (RNA) extraction is an essential technique in molecular biology studies which uses refined processes to isolate RNA out of biological material such as blood. The process should be carried out timeously and in an environment that is specially prepared for

the isolation by using RNase degrading agents to wipe down surfaces and remove readily available RNases. There are various methods of isolation which include organic, spin basket format, direct lysis and magnetic particle methods (Vomelová et al., 2009). The protocol selected for the purpose of this study used the spin basket format method which uses membrane filters to separate RNA from biological matter.

Total RNA was extracted from fresh whole blood using the QIAamp® RNA Blood Mini kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instruction. Each blood sample was transferred in 0.5 ml, 1.5 ml or 1 ml volume into a 15 ml falcon tube containing buffer EL; measured as 5 times the starting blood volume. Samples were incubated on ice for a maximum period of 15 minutes; followed by centrifugation with centrifuge 5810R (Eppendorf Hamburg, Germany) at 1890 rpm for 10 minutes at 4ºC. The supernatant was discarded and buffer EL was added to the pellet in a measure of 2 volumes of the starting blood sample; the tubes were vortexed briefly and samples were centrifuged at 1890 rpm for 10 minutes at 4ºC. The supernatant was discarded; and the pellet was re-suspended in buffer RLT containing 0.1% 2-mercaptoethanol. The resulting lysate was transferred into a QIAshredder spin column in a 2ml collection tube; each sample was centrifuged with centrifuge 5424 (Eppendorf, Hamburg, Germany) at 14 000 rpm for 2 minutes.

Following centrifugation, the QIAshredder spin column was discarded and the lysate was collected; to which 1 volume of 70% ethanol was added and mixed by pipetting. The lysate was carefully transferred into a new QIAamp spin column in a 2 ml collection tube; the samples were centrifuged at 10 000 rpm for 15 seconds. The collection tube and supernatant were discarded; and the spin column was placed into a new 2ml collection tube.

Buffer RW1 was added in a measure of 350µl into the column and the sample was centrifuged for 15 seconds at 10 000 rpm; the supernatant was discarded, and the tube was reused. A DNase digestion master mix (50 µl of 2X DNase 1U/L buffer, 8 µl of reconstituted DNase, 10 µl Manganese Chloride solution and 32 µl Nuclease free water) was prepared separately. The DNase master mix was aliquoted into each tube in 100 µl volume; the samples were incubated at room temperature for 15 minutes.

Following incubation, 350 µl of buffer RW1 was added onto the column and the samples were centrifuged at same conditions as the previous step. The resulting supernatant was discarded together with the collection tube and the column was placed into a new collection tube. Buffer RPE was added into the column in a volume of 500 µl and the tubes were centrifuged at 10 000 rpm for 15 seconds; the supernatant and collection tube were both discarded. The column was

placed into a new collection tube. The previous step was repeated with centrifugation conditions set at 14 000 rpm for 3 minutes; thereafter the supernatant and collection tube were discarded and the column was placed into a new collection tube.

The tube was centrifuged at 14 000 rpm for 1 minute and the column was placed into a clean 1.5 ml microfuge tube. RNase- free water was added onto the centre of the column to a maximum volume of 50 µl; the tube was incubated at room temperature for 5 minutes and thereafter it was centrifuged at 10 000 rpm for 1 minute. Total RNA quantity and integrity were measured on the Nanodrop ND 1000 Spectrophotometer (Thermo Fisher Scientific, Weltham, Massachusetts, United States) and Agilent® 2100 Expert Bioanalyzer™ (Agilent Technologies, Santa Clara, California, United States) with the Agilent® RNA 6000 Nano system at the Central Analytical Facility (CAF), Stellenbosch University all samples were stored at -80^{o}C.

3.4. DNA extraction

Deoxyribonucleic Acid (DNA) extraction is also essential like the isolation of RNA; however, DNA is very stable and robust compared to RNA, as such it does not require immediate isolation. DNA isolation employs numerous methods which include solid phase, Chelex or organic extractions (Elkins, 2013). All these methods act by isolating DNA from biological samples and making it readily available for downstream processing. In this study organic extraction method was employed to extract DNA from whole blood.

Genomic DNA was extracted from whole blood using an adaptation of the Miller et al 1988 protocol. Genomic DNA was extracted by a combination of two techniques: salt lysis and alcohol precipitation. Red blood cell (RBC) lysis was carried out by transferring 500 µl of blood sample into 2 ml Eppendorf tube containing 1ml cold lysis buffer (0.155 M NH_4CL, 0.01 M $KHCO_3$ and 0.0001 M EDTA); the solution was briefly vortexed and then centrifuged with centrifuge 5424 (Eppendorf, Hamburg, Germany) at 14 000 rpm for 15 minutes. The supernatant was carefully discarded and another 1 ml of cold lysis buffer was added to the pellet; which was vortexed and centrifuged at 14 000 rpm for 15 minutes. The supernatant was carefully discarded and the pellet was re-suspended with 1ml cold phosphate buffer saline (tablet) and vortexed briefly. The samples were centrifuged at the same conditions as the previous step and the resulting supernatant was carefully discarded. The pellet was re-suspended with 500 µl cold lysis buffer (0.01 M Tris, 0.4 M NaCl and 0.002 M EDTA), 10%

SDS and 3 µl 20 mg/ml Proteinase K (Thermo Fisher Scientific, Weltham, Massachusetts, United States); the samples were incubated at 56°C overnight.

To precipitate the DNA, 200 µl of 6M NaCl was added to the tube containing the overnight sample and solute mixture; and then vortexed for 15 seconds. The samples were centrifuged at 5 000 rpm for 15 minutes and the supernatant was transferred into a clean 1.5 ml Eppendorf tube. One volume of isopropanol was added to the supernatant and the tubes were inverted gently until the solution was clear; the samples were then incubated at -80ºC for 30 minutes. DNA was then pelleted by centrifugation at 14 000 rpm for 30 minutes and the pellet was washed with 100 µl of 70% Ethanol followed by centrifugation at 14 000 rpm for a further 15 minutes. The pellets were dried briefly at room temperature and then re-suspended in a final volume of 30µl nuclease free water. The concentration of DNA yielded was quantified using a Nanodrop ND 1000 Spectrophotometer (Thermo Fisher Scientific, Weltham, Massachusetts, United States) and the samples were immediately stored at -20°C for future use.

3.5. Reverse Transcription and cDNA synthesis

In order to study RNA downstream, a conversion from RNA to a complementary DNA (cDNA) strand is required since amplification methods works with the double stranded DNA. The RNA provides a template from which the cDNA is reverse transcribed by the enzyme reverse transcriptase. RNA concentrations determined with Agilent® 2100 Expert Bioanalyzer™ (Agilent Technologies Santa Clara, California, United States) were used to calculate the starting RNA volume in order to have starting concentration of 100ng/µl.

Residual genomic DNA was removed using the QuantiNova™ Genomic DNA removal components (Qiagen, Venlo, Netherlands) according to the manufacturer's manual. Each RNA sample was aliquoted into a 200 µl tube in a 100 ng/µl concentration containing (2 µl of gDNA removal and RNAse inhibitor mix) made up to 15 µl with RNase-free water. The samples were incubated at 45ºC for 2 minutes then placed on ice immediately to remove excess genomic DNA.

Complimentary DNA (cDNA) was synthesized using the QuantiNova™ Reverse Transcription Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol. A master mix was prepared containing 1 µl of the Reverse transcription enzyme and 4 µl of reverse transcription mix for each sample; which was added to the genomic removal reaction. The samples were placed on the 2720 Thermal cycler (Applied Biosystems, Scientific, Weltham, Massachusetts,

United States); cDNA synthesis occurred under the following cycling conditions, annealing at 25ºC for 3 minutes, reverse transcription at 45ºC for 10 minutes and enzyme inactivation at 85ºC for 5 minutes. The cycler's lid was kept open to avoid vaporization; thereafter the samples were immediately placed on ice.

3.6. Real Time Quantitative Polymerase Chain Reaction

The use of real time quantitative polymerase chain reaction (RT-qPCR) in molecular biology has become an important tool with various applications including disease diagnosis. RT-qPCR is an essential quantitative method that is used to detect the expression of RNA transcripts through two techniques namely one-step or two-step reactions. In one-step reaction the reverse transcription and quantification occurs sequentially in one tube under optimized reaction conditions, whereas two-step reaction has the reverse transcription and quantification occurring in separate tubes under optimized conditions (Santos et al., 2004). For the purpose of this study the two-step technique was used.

RT-qPCR was carried out on the ABI 7900HT Fast Real Time PCR system (Applied Biosystems, Weltham, Massachusetts, United States); amplification was performed using the QuantiNova™ SYBR® Green RT-qPCR kit (Qiagen Venlo, Netherlands). QuantiTect® Primer Assays (*RET* ENSG00000165731, *EDNRB* ENSG00000136160, *SOX10* ENSG00000100146 and *NRG1* ENSG00000157168) and the endogenous controls *HPRT* ENSG00000165704 and HSP (Qiagen Venlo, Netherlands) were used for amplification according to the manufacturer's protocol. The components of the RT-qPCR are listed in table 3.2. Cycling conditions for RT-qPCR were set as follow: heat activation at 95ºC for 2 minutes, 40 cycles of 2-step cycling: denaturation at 95ºC for 5 seconds and annealing/extension at 50ºC for 10 minutes. All sample reactions were performed in triplicate.

Table 3.2: RT-qPCR components and volume of each component used for the real time amplification of the HSCR cohort samples

Component	384 well volume	Final Concentration
2x QuantiNova SYBR Green RT-PCR Master Mix	5 µl	1x
QN ROX Reference Dye (Applied Biosystems cycler only)	0.05 µl	1x
10x primer mix	1 µl	1x
Template RNA	Variable	100 ng/µl reaction
RNase-Free Water	Variable	-
Final Volume	10 µl	

3.7. Polymerase Chain Reaction (PCR)

Standard PCR is a technique used for the amplification of DNA under the action of the enzyme DNA polymerase. It uses one strand of the DNA as a template meanwhile the enzymes adds deoxyribonucleotide triphosphates (dNTPs) to the growing strand. This method is widely to amplify specific regions in the genes of interest for various studies such as mutational analysis.

Following genomic DNA extraction, the protein coding exons were amplified with primers designed to anneal to each of the 8 *ACTG2* exons. Primers were designed using the PrimerQuest and OligoAnalyzer 3.1 tools (Integrated DNA Technologies®) (Table 3.3). The components for the PCR and their respective concentrations are shown in table 3.4. PCR cycling was carried out on the 2720 Thermal Cycler (Applied Biosystems, Weltham, Massachusetts, United States) with the following parameters; 94°C for 5 minutes initialization, 30 cycles of denaturation at 94°C for 30 seconds, annealing at 50-62°C for 30 seconds and extension at 72°C for 30 seconds, followed by final extension at 72°C for 7 minutes and an infinity hold at 4°C.

Table 3.3: *ACTG2* exons primers designed on the Oligo Analyzer platform, showing the optimised annealing temperature of each exon primer set

Primer	Sequence	Purification	TA
ACTG2-3F	TTC ACA TTT CAG GGC AGA GG	25nm	52°C
ACTG2-3R	GCTCAAAGCCTGGTGGTAT	25nm	
ACTG2-4F	GTCTCCTGCTATCCTGTTTCTG	25nm	58°C
ACTG2-4R	TGCAATAGTCCAGGGAGAGA	25nm	
ACTG2-5F	GATCCATCCCATCCTGTGTAAC	25nm	59°C
ACTG2-5R	GGCATGGACCACAGACATAG	25nm	
ACTG2-6F	GGGAGTGGGTGTGGAATAAT	25nm	58°C
ACTG2-6R	CTATACCAGCTAGGCTCACATC	25nm	
ACTG2-7F	GGTAGTCAGAGCTCATTGGTAAC	25nm	62°C
ACTG2-7R	GTCCTGAGAACTTCTTGTCCTAA	25nm	
ACTG2-8F	GGTTGCAGTGAGCCAAGATAG	25nm	60°C
ACTG2-8R	CATGACTCCTGGTGTTTCTCTC	25nm	
ACTG2-9F	CGAAGAAGGGTCATTTGAGGAG	25nm	61°C
ACTG2-9R	CAATATCATCCTGGACTGGAGC	25nm	
ACTG2-10F	TGGACCACCTTGCTTATTCC	25nm	56°C
ACTG2-10R	CCCACACAGAGAAGTAAGGC	25nm	

T_A= Annealing temperature

Table 3.4: Components used in the PCR amplification of the *ACTG2* exons for both HSCR and ADL cohorts

Component	Volume per reaction	Final concentration
10x Super Therm Gold Taq Buffer	12.5 µl	5x
10mM dNTPs	2 µl	0.2 mM each
5U/µl Super Therm Gold Taq	0.075 µl	0.5U
Forward primer	1.5 µl	10µM
Reverse primer	1.5 µl	10µM
Template DNA	Variable	100 ng/µl
Nucleated water	Variable	-
Final Volume	25 µl	

3.8. Agarose gel electrophoresis

Agarose gel electrophoresis is a widely used method of visualizing biological macromolecules which employs intercalating dyes to enable visualization. It separates DNA/RNA according to size while using the electric current applied on the gel to move the molecules, the smaller fragments migrate quicker and end up at the bottom of the gel and the larger molecules migrate slower and occupy the top region of the gel. This technique is used as a qualitative method to either check for amplification post PCR or the size of fragments digested and undigested. Here it was used to check for successful amplification post PCR.

Post amplification, the samples were checked for successful amplification by gel electrophoresis at 100V on a 1% agarose gel prepared with 1x SB (di-sodium tetraborate decahydrate) buffer and stained with 1.5 µl ethidium bromide. Samples were loaded onto the prepared gel in 4 µl volume with 2 µl of 6x DNA Gel loading dye (ThermoFischer Scientific, Weltham, Massachusetts, United States). A 1kb DNA ladder (Kapa biosystems, Salt River, Cape Town, South Africa) loaded with the samples; added in 2.5 µl volumes. Visualisation of the gels was carried out on the GeneSnap software (SYNGENE, India) under ultraviolet light illumination.

3.9. Sequencing

To determine the order of the nucleotides on a gene of interest sequencing is carried out on the PCR product which is subjected to a detection by synthesis method known as Sanger

sequencing. The nucleotide bases give off fluoresce upon incorporation into the complimentary strand of the cloned gene segment which is detected and read as a chromatograph.

Samples that were successfully amplified with the PCR technique were purified and sequenced bidirectionally on the ABI 3730xl/3500xl DNA Sequencer (Applied Biosystems, Weltham, Massachusetts, United States) at CAF and Inqaba biotech using the *ACTG2* primers spanning the intron-exon boundaries. The sequences were analysed on the FinchTV 1.4.0 chromatograph viewer (Geospiza, Inc.).

3.10. *In Silico* analysis

3.10.1. Pathogenicity prediction

Prediction tools are used in research to further determine the effects of novel SNPs *in silico*, the algorithm of the tools use scores to rank SNPs based on sequence conservation, genic effects and regulatory element annotations (Rentzsch et al., 2019). Numerous tools are available which include but not limited to Sorting Intolerant From Tolerant (SIFT), PolyPhen-2, MutationTaster and Combined Annotation Dependent Depletion (CADD), newly identified SNPs are considered significant or non-significant based on these prediction scores. Where benign is classified as score from 0-0.68, possibly damaging from 0.7-0.89 and probably damaging from 0.9-1.00 by PolyPhen-2. CADD uses a scaled score known as PHRED-like which is a $\log_{10}$ where benign is any value from 0-15 and deleterious is a value above 20 (Kircher et al., 2014; Rentzsch et al., 2019). Meanwhile SIFT utilizes sequence homology within species in order to predict if a nucleotide base exchange will affect the resulting protein function, its scoring method categorizes SNPs as either tolerated with a score greater than 0.05 or deleterious with a score equal or less than0.05 (Pauline and Henikoff, 2003).

Novel *ACTG2* variants observed on the sequences were tested on the Hope 1.1.1 (Center for Molecular and Biomolecular Informatics) http://www.cmbi.ru.nl/hope for changes in the amino acids. They were further tested for pathogenicity on PolyPhen-2 http://genetics.bwh.harvard.edu/pph2/, MutationTaster http://www.mutationtaster.org, SIFT http://sift.bii.a-star.edu.sg/www/SIFT_seq_submit2.html and CADD https://cadd.gs.washington.edu/snv; the species conservation of the *ACTG2* was tested on the CLUSTALW (Kyoto University Bioinformatics Center) platform.

3.10.2. Secondary structure prediction

SWISS MODEL (The Center for Molecular Life Sciences, University of Basel) https://swissmodel.expasy.org/interactive was used to model the variants observed on the *ACTG2* exons and visualize the possible structural change on the protein. Additionally, Self-Optimized Prediction from Multiple Alignment (SOPMA) (Institut de Biologie et de Chimie des Protéines, UPR 412-CNRS, Lyon, France) https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html was used to predict the secondary structure modifications of the mutants.

3.10.3. Human Splicing Finder

In order to study the potential effect of SNPs occurring in the intronic boundaries of eukaryotic cells, the Human Splicing Finder (HSF) algorithm was developed (Ohno et al., 2018). It's function of assigning the significance of splice sites is based on categorizing them into one of the 2 groups; namely exonic splice silencer (ESS) which are cis-regulatory elements that inhibit use of adjacent splice sites or exonic splice enhancer (ESE) which promote regulated and constitutive splicing (Desmet et al., 2009; Grodecká et al., 2017). Homozygous variants that were observed in the intronic regions of *ACTG2* were tested for possible interfering with the splice sites of the gene using HSF (Bioinformatics & Genetics Team, Aix Marseille Université) http://www.umd.be/HSF3/.

3.10.4. PyMOL

Visualization of compounds is essential in the study of macromolecules especially in the prediction of bonds between compounds. PyMOL provides such a platform for molecular docking, binding site analysis and 3D virtual analysis of biological macromolecules (Chaudhari and Li, 2015; Seeliger and de Groot, 2010). The identified variants were modelled on the PyMOL 2.2 (Schrödinger, Inc.) to further predict their effect on the protein structure.

CHAPTER 4

4. Results

4.1. HSCR ENS genes

4.1.1. Preliminary mapping of candidate genes

Genes selected for the study of quantitative expression in the HSCR cohort were shown to be co-expressed in the gene interaction network generated with GENEMania, illustrated by figure 4.1. *EDNRB* is associated with melanocyte development; it is shown to be co-expressed with both *SOX10* and *RET* in figure 4.1 *SOX10* is also linked to the development of melanocyte. Whereas, *NRG1* which is known to be involved in melanocyte development does not share a pathway with either *SOX10* and *EDNRB*, resulting from their localization within the cell. *NRG1* is located within the mesenchyme whereas *EDNRB* and *SOX10* are both located in the nucleus (figure 2.3). Thus, *NRG1* mechanism of action in melanocyte development may be achieved through a different pathway.

Whilst *SOX10* has been shown to have a direct effect on both *EDNRB* and *RET* as a transcription factor, none of the genes of interest on figure 4.1 are shown to directly share biological pathways. Co-expression of these genes is linked to their biological processes in ENS development, although they achieve it through different pathways.

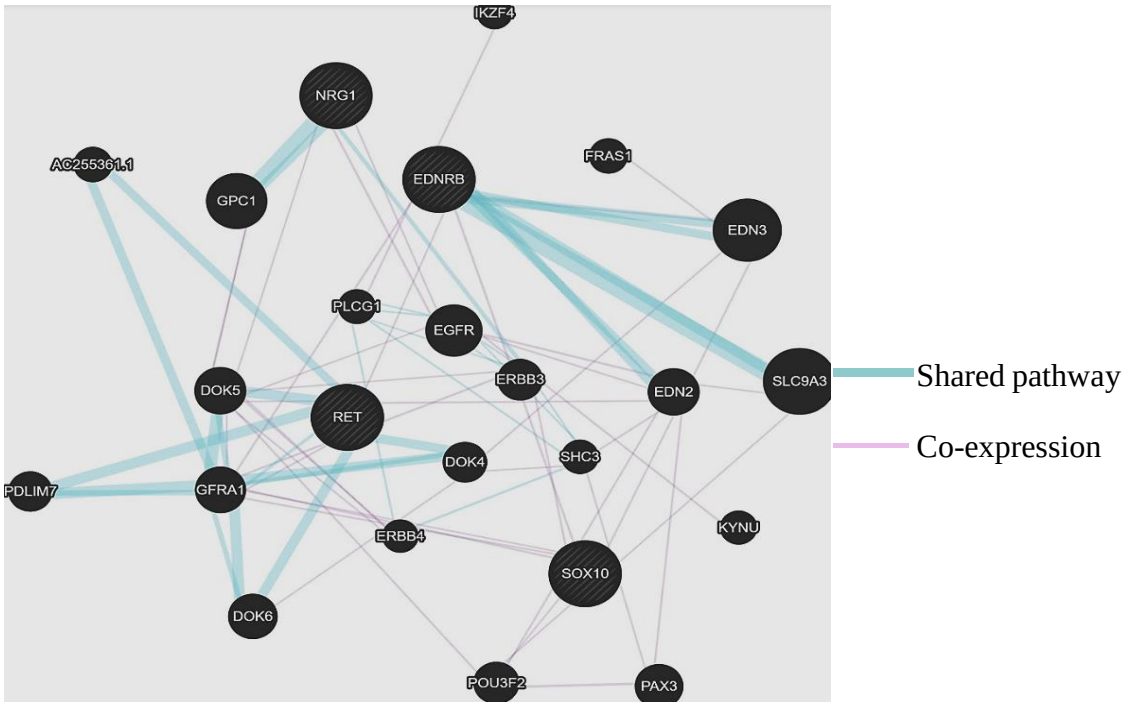

Figure 4.1: Protein association network of the HSCR genes of interest generated with GENEMania, showing the genes co-expression and shared pathways

4.1.2. Quantitative expression

Eight samples were chosen for RT-qPCR amplification, these were samples with RNA concentration above 80 ng/µl and RNA integrity (RIN) value above 7 based on the Bioanalyzer and Nanodrop RNA quantification shown in table 4.1. Protocol optimization of the RT-qPCR was performed using RNA sample not from the study cohort, by amplification of the genes of interest (*EDNRB*, *SOX10*, *NRG1* and *RET*) under the conditions outlined in Chapter 3, 3.5 and 3.6. Following this result, the first run of the RT-qPCR was carried out; and the temperature cycling worked, however further optimisation was required to successfully amplify the patient samples.

Figure 4.2 illustrates an amplification plot of the selected HSCR genes of samples HD6,9-14 showing the number of cycles on the x axis and the reporter value on the y as a log- measure. From the entire sample set only 2 samples reached the amplification threshold within 18 cycles; the other samples reached their threshold well after 30 cycles. The amplification plot shows non-specific binding; contrary to what is expected of a successful RT-qPCR; it produced data with very high cycle threshold (C_t) values as illustrated by figure 4.2. Repeats of the protocol with increased sample RNA, yielded the same results. RNA samples HD14-17 were not quantified on the Bioanalyzer (table 4.1).

Table 4.1: HSCR samples showing the RNA concentration quantified with the NanoDrop and Bioanalyzer machines and their respective RIN value

Sample	Concentration (NanoDrop)	Concentration (Bioanalyzer)	RIN value
HD03	157.6ng/µl	126.8 ng/µl	2.7
HD06	139.8 ng/µl	178 ng/µl	10
HD07	23.6 ng/µl	37 ng/µl	9.80
HD08	50.0 ng/µl	49 ng/µl	9.30
HD09	72.4 ng/µl	98 ng/µl	9.70
HD10	88.0 ng/µl	85 ng/µl	10
HD11	78.5 ng/µl	101 ng/µl	10
HD12	101.7 ng/µl	83 ng/µl	10
HD13	160.8 ng/µl	217 ng/µl	10
HD14	303.1 ng/µl	-	-
HD15	39.6 ng/µl	-	-
HD16	38.6 ng/µl	-	-
HD17	257.1 ng/µl	-	-

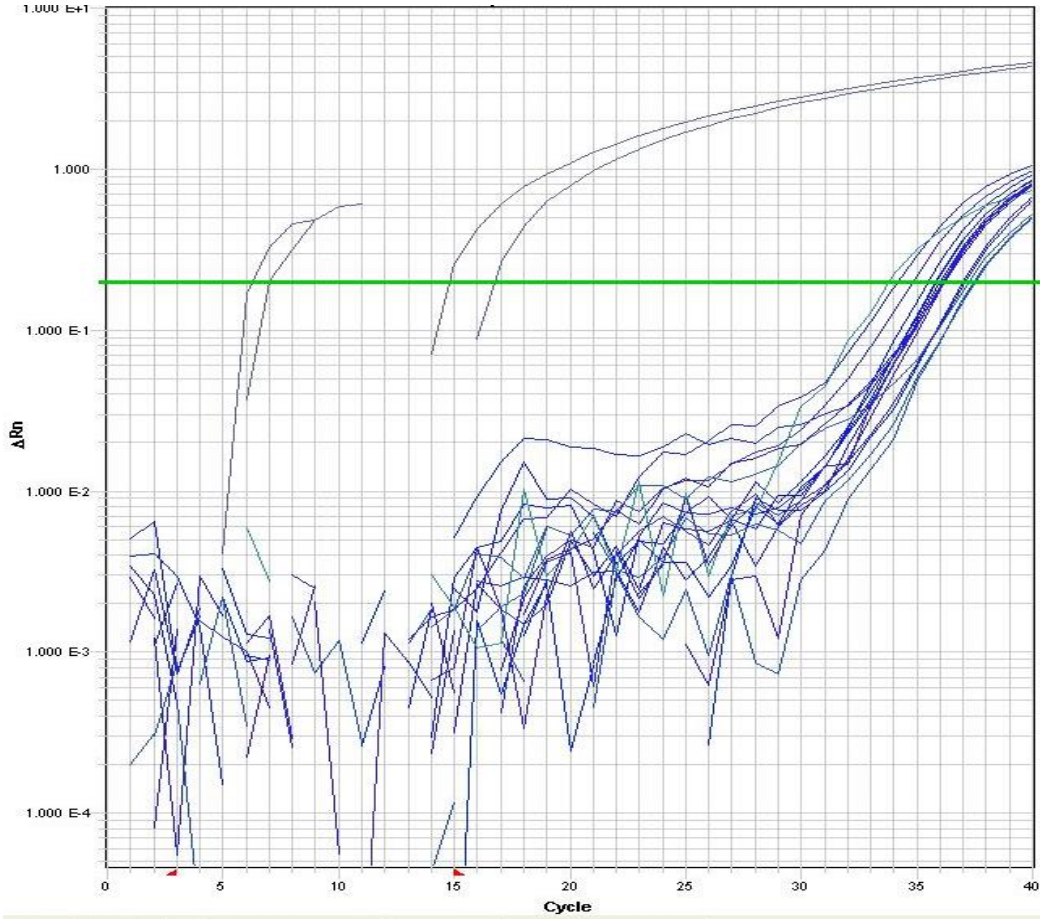

Figure 4.2: RT-qPCR amplification diagram of the HSCR cohort illustrating the gene expression of the HSCR cohort as number of cycles on the x-axis and the normalized reporter value on the y-axis as a log measure

4.2. Smooth muscle

HSCR cohort

4.2.1. Smooth muscle dysmotility in HSCR patients

Whole blood samples were collected from a total of 17 HSCR patients consisting of 13 males and 3 females. Fourteen samples (13 males and 1 female) were screened for *ACTG2* mutations; the other 3 samples were excluded based on the low DNA concentration and quality as determined by the Nanodrop quantification. Variants identified in the study cohort were single base non-synonymous exchanges occurring in the 8 protein coding exons (exon 3-10) such as the one shown in figure 4.3; these were studied further *in silico* to investigate their effects on the ACTG2 protein model. Figure 4.3 shows a chromatograph of one of the HSCR patients with the *ACTG2* S345L mutation shown by the dotted box around a homozygous SNP, the variant shows both C and G codons occurring at the same position.

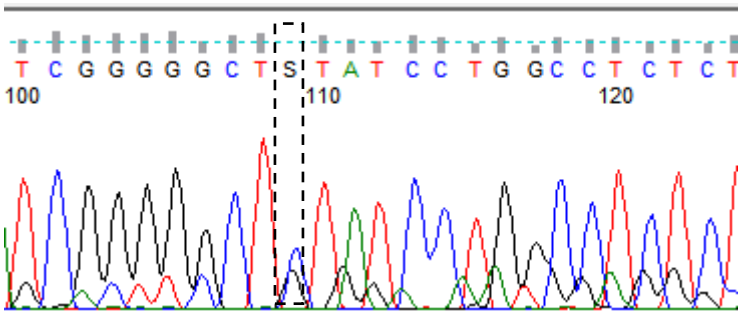

Figure 4.3: Amino acid chromatograph showing the S345L mutation occurring on the *ACTG2* exon 10 of one of the HSCR patients analysed on FinchTV

ACTG2 variants observed in the HSCR cohort are outlined in table 4.2; these included known and new amino acid coding and intronic variants. c>t -IVS6 on exon 3 and T>G V160R on exon 7 are known variants classified as SNPs without clinical significance by Single Nucleotide Polymorphism database (dbSNP) rs1050146 and rs761385269 respectively; T>G V160R was sequenced on the reverse strand with the annotation V160L. Newly identified SNPs were considered significant or non-significant based on the SIFT, PolyPhen-2 and CADD pathogenicity prediction scores. Where benign 0-0.68, possibly damaging 0.7-0.89 and probably damaging 0.9-1.00 by PolyPhen-2. Deleterious >20> benign by scaled CADD, and deleterious <0.05< tolerated by SIFT shown on table 4.4. Intronic SNP g>c -IVS12 which occurred on exon 3 was observed in 4 patients, this SNP was studied further *in silico* and

classified as a polymorphic SNP by MutationTaster. It was predicted to modify exonic splice site by both MutationTaster and HSF; its effect was predicted to create an exonic splice silencer which shifts the splice site.

Other variants, L217A and L243P were considered non-significant as a result of the PolyPhen-2 scores 0.508 and 0.172 respectively shown in table 4.4. Although L217A was also placed within the 10% most deleterious variants by the scaled CAAD score of 10.73. L243P variant was observed in 1 patient whereas L217A was identified in 2 patients. Another variant considered non-significant was the V153C variant observed in 2 patients of the study cohort. It was classified as pathogenic by both PolyPhen-2 and SIFT; it may be significant considering these scores, although CADD ranked it as non-pathogenic.

Meanwhile, nonsynonymous amino acid change from a lysine (Lys/K) to glutamic acid (Glu/E) or arginine (R) on amino acid residue position 119 (K119E/R) were observed in the majority of the samples; 9 out of 13 patients and 6 out of 9 control samples (tables 4.2 and 4.3). The SNP was predicted to be benign by PolyPhen-2 score of 0.373; however, it was predicted to be within the top 10% of most deleterious SNP by CADD with a score 13.43. In addition, SIFT predicted the SNP to be pathogenic as shown in table 4.4 with a 0.00 score and a median species conservation score of 3.15; this may be the consequence of the high sequence conservation in the ACTG2 protein amongst species as shown by the species conservation phylogenetic tree (appendix figure 7.1) predicted by the CLUSTALW platform. Moreover, at position 119 another variant exists K119Q which has been identified and characterised on dbSNP rs747545226; the variant is predicted to be of no clinical significance.

Table 4.2: Known and novel *ACTG2* variants observed in the HSCR cohort

Sample	Exon3	Exon4	Exon5	Exon6	Exon7	Exon8	Exon9	Exon10
HD03	WT	WT	c.171 A>G K119E	WT	WT	c.221G>C L217A	WT	c.108 T>G W357G
HD05	c.134 g>c -IVS12	WT	c.171 A>G K119E	WT	WT	c.221G>C L217A	WT	c.109C>G S345L
HD06	c.134 g>c -IVS12	WT	c.171 A>G K119E	WT	WT	WT	WT	c.109C>G S345L
HD07	WT	WT	WT	WT	WT	WT	WT	WT
HD08	c.134 g>c -IVS12	WT	c.171 A>G K119E	WT	c.81T>G V160R rs761385269	WT	WT	c.109C>G S345L
HD09	WT	WT	c.171 A>G K119E	WT	WT	WT	WT	c.109C>G S345L
HD10	WT	WT	WT	WT	WT	WT	WT	c.109C>G S345L
HD11	c.74 c>t -IVS6 rs1050146	WT	c.171 A>G K119E	WT	WT	c.300 G>C L243P	WT	c.109C>G S345L
HD12	WT	WT	WT	WT	WT	WT	WT	WT
HD13	WT	WT	c.175 A>G K119R	WT	c.62 T>C V153C	WT	WT	WT
HD14	c.74 c>t -IVS6 rs1050146	WT	c.171 A>G K119E	WT	WT	WT	WT	WT
HD15	c.74 c>t -IVS6 rs1050146	WT	c.171 A>G K119E	WT	WT	WT	WT	WT
HD16	WT	WT	WT	WT	WT	WT	WT	WT
HD17	c.134 g>c -IVS12	WT	WT	WT	c.62 T>C V153C	WT	WT	WT

rs =accession number, dbSNP
WT= Wild Type
> = base exchange/substitution

Furthermore, the variant K119E/R was also observed in the healthy control cohort (figure 4.3); supporting the rationale that the variant is possibly not directly involved in the disease pathogenesis. It may be essential to the enteric smooth muscle pathophysiology as a disease modifier instead; considering that all the HSCR patients observed with possibly pathogenic variants also carried this variant. Figure 4.4 depicts a modelled tertiary structure of the variant K119E; showing no possible effect on the other amino acids within close bonding distance on the tertiary structure of ACTG2. The amino acid exchange is non-conservative changing a basic residue Lys to a polar uncharged residue Glu; predicted to have little to no effect on the protein-protein interaction. The healthy control cohort also had the intronic SNP c>t -IVS6 rs1050146 determined by literature to be clinically insignificant.

Table 4.3: ACTG2 screening of the healthy control samples observed to have the intronic C>T -IVS6 and K119E/R variants

Sample	Exon3	Exon4	Exon5	Exon6	Exon7	Exon8	Exon9	Exon10
NC1	WT	WT	c.174 A>G K119E	WT	WT	WT	WT	WT
NC2	c.74 C>T -IVS6 rs1050146	WT	WT	WT	WT	WT	WT	WT
NC3	WT	WT	c.176 A>G K119R	WT	WT	WT	WT	WT
NC4	c.74 C>T -IVS6 rs1050146	WT	WT	WT	WT	WT	WT	WT
NC5	WT	WT	WT	WT	WT	WT	WT	WT
NC6	WT	WT	c.176 A>G K119E	WT	WT	WT	WT	WT
NC7	WT	WT	c.176 A>G K119E	WT	WT	WT	WT	WT
NC8	WT	WT	c.176 A>G K119E	WT	WT	WT	WT	WT
NC9	c.74 C>T -IVS6 rs1050146	WT	c.176 A>G K119E	WT	WT	WT	WT	WT

rs =accession number, dbSNP
WT= Wild Type
> = base exchange/substitution

Two SNPs of interest observed in the HSCR cohort namely T>G W357G and C>G S345L were both observed in exon 10 of *ACTG2*; both SNPs were predicted to be damaging by all the prediction networks used as shown in table 4.4. Based on this they were studied further *in*

silico to elucidate the effects they may have on the ACTG2 protein and possibly the enteric smooth muscle etiopathogenesis in Hirschsprung's disease patients.

Table 4.4: *ACTG2* single nucleotide polymorphisms (SNPs) identified in HSCR patients showing the pathogenicity score as predicted by the nsSNP predictor tools, the number of patients affected and the exon which each variant occurs

Variant	Exon	#	PolyPhen-2	SIFT (msc)	(CADD)
K119E/R	5	9	0.373	0.00 (3.15)	13.43
V153C	7	2	1.00	0.00 (3.15)	4.6
V160R	7	1	0.74	0.05 (3.15)	6.73
L217A	8	2	0.508	0.00 (3.15)	10.73
L243P	8	1	0.172	0.00 (3.15)	4.5
S345L	10	6	0.99	0.00 (3.15)	25.8
W357G	10	1	0.97	0.00 (3.12)	33

*msc = Median sequence conservation

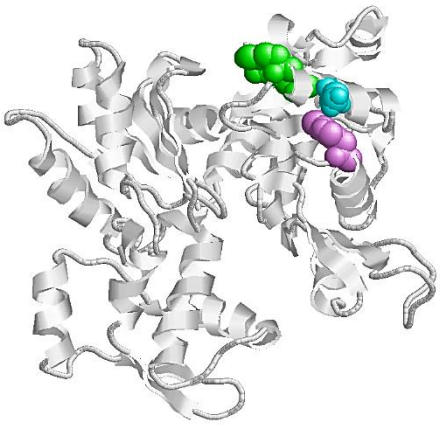

Figure 4.4: Tertiary structure modelling of the *ACTG2* K119E variant and the neighbouring amino acid residue in close bonding distance; showing the variant Glu in cyan, Lys at position 114 and 117 in green and Trp at position 80 in magenta generated with PyMOL

4.2.2. Secondary structure predictions

ACTG2 SNPs S345L and W357G were not present in the control sample set and were thus chosen for further analysis. In silico analysis with scaled CADD PHRED of S345L and W357G resulted in pathogenicity prediction scores of 25.8 and 33 respectively presented in table 4.3, thus the variants were predicted to be probably disease causing based on these scores. S345L

variant occurred by a homozygous single base substitution as illustrated by the boxed in region of figure 4.3. *In silico* modelling with SWISS model predicted structural changes on the non-synonymous mutation S345L where the wild type is shorter as depicted on figure 4.5 A compared to the longer side chain of the mutant shown in figure 4.5 B. This SNP was observed in 46% (6 out of 14) of the study cohort whereas the control samples were all wild type.

Furthermore, the mutation resulted in an altered secondary structure of the protein by substitution of an uncharged; polar Serine (Ser/S) with a non-polar hydrophobic Leucine (Leu/L). The substitution from Ser to Leu introduces an amino acid change from a neutral and small amino acid into a hydrophobic residue, this change alters the secondary structure due to the properties of Leu which prefers to be buried within the core of the protein unlike Ser. Amino acid Ser is located on a helical structure on the ACTG2 protein; hence, changing the residue may affect the protein folding into its tertiary structure and subsequently the quaternary structure. Secondary structure prediction with SOPMA showed that the wild type has a coil which is adjacent an alpha (α) helix on the protein sequence; meanwhile the mutant produces an extended strand instead.

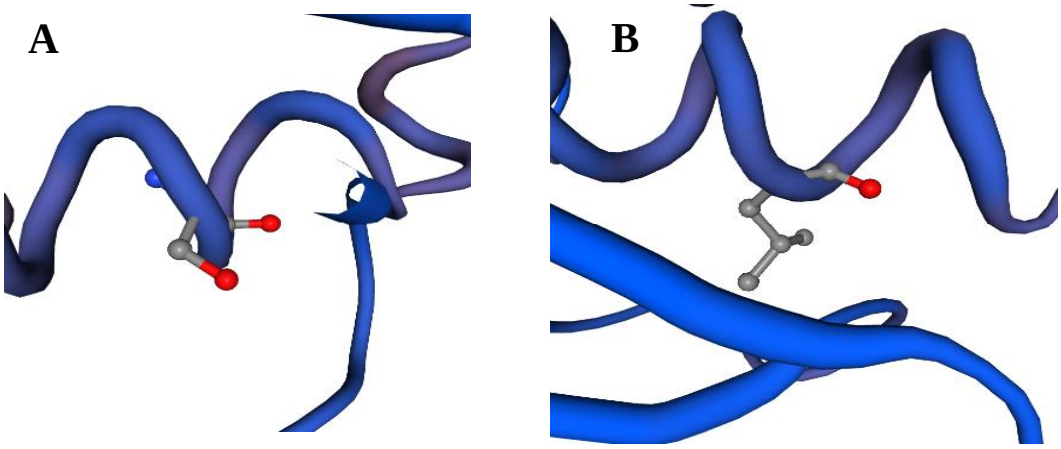

Figure 4.5: Protein structure prediction model of the *ACTG2* variant S345L generated with SWISS Model showing the wild type with a shorter side chain (**A**) and the mutant with an extended side chain (**B**) also showing the amino acids Ser and Leu localization on the protein

A heterozygous base change from T to G on exon 10 shown in figure 4.6 resulted in a variant W357G in one of the patients. W357G is also a non-conservative variant which has an amino

acid replacement from an aromatic Tryptophan (Trp/W) to a small Glycine (Gly/G); this amino acid change subsequently changes the conformation from a short beta (β) strand into a β-turn as predicted by SOPMA.Figure 4.7 illustrates the modelled structure of the wild type **A** and variant **B**; the aromatic ring of the wild type Trp is substituted by a Gly in the mutant which is considerably small compared to the Trp. Secondary structure prediction of the ACTG2 protein also showed the amino acid W to be preceded by an α-helix that is followed by a coil, these are abolished by the amino acid residue substitution.

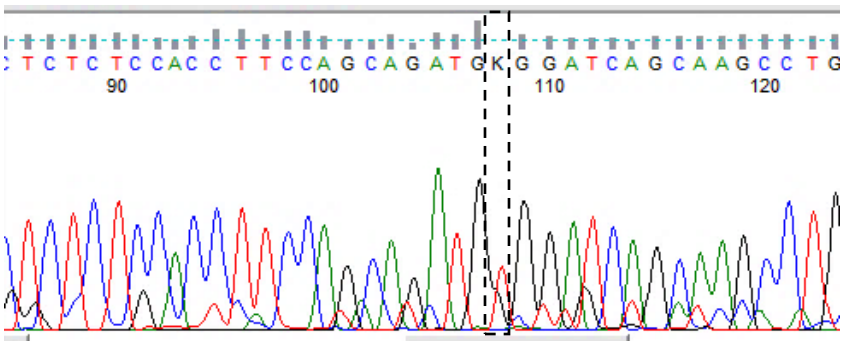

Figure 4.6: Amino acid chromatograph illustrating a heterozygous base exchange T>G on *ACTG2* exon 10 producing the W357G variant analysed on FinchTV

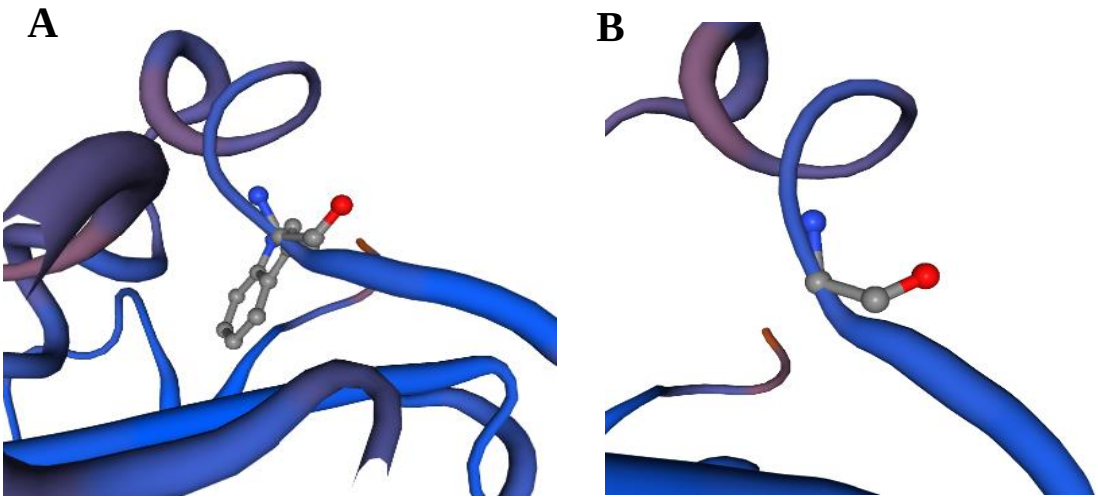

Figure 4.7: Protein modelling of the *ACTG2* variant W357G showing the wild type with an aromatic side chain Trp (**A**) and the mutant with a short Gly side chain (**B**) generated with SWISS Model

Furthermore, Trp is an aromatic residue known to carry pi (π) electrons which interact in covalent bonds such as ring stacking with other aromatic residues or cation bonds with basic residues; the bonds are therefore affected by non-conservative amino acid substitution due to the π electrons reactivity. Residue interaction within bonding distance of the Trp wild type showed it to have close interaction with Phenylalanine (F) on position 353 and Tyrosine (Y) on position 134 illustrated in figure 4.8. Y134 residue is a known visceral myopathy variant . Taken together these data suggest that the mutation W357G may have a significant effect on the aetiology of the enteric smooth muscle in Hirschsprung's patients carrying this mutation.

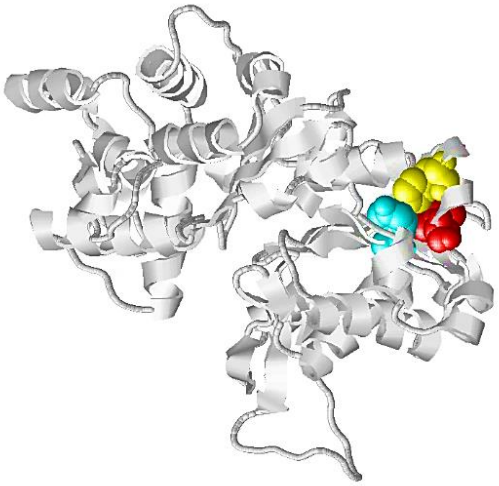

Figure 4.8: Modelled secondary structure of ACTG2 protein generated with PyMOL showing the wild type aa residue W on position 357 in red and neighbouring amino acid residues F353 in yellow and Y134 in cyan within bonding distance of each other

ADL cohort

4.2.3. Smooth muscle degeneration in ADL patients

Variants observed in the 5 ADL patients did not include known Visceral SCM SNPs; table 4.5 displays all the variants identified in the ADL cohort including the exon in which they occur. Variant K119E/R was also identified in the ADL patients occurring together with homozygous amino acid base exchange occurring at the intronic regions (figure 4.9). The variant K119E/R occurring on exon 5 was observed in 3 patients which also had the intronic variants t>c-IVS3 on the same exon/intron boundary and t>c +IVS16 post exon 7. Meanwhile g>c -IVS12 also occurred in 3 patients together with t>c +IVS16 variant in 1 patient.

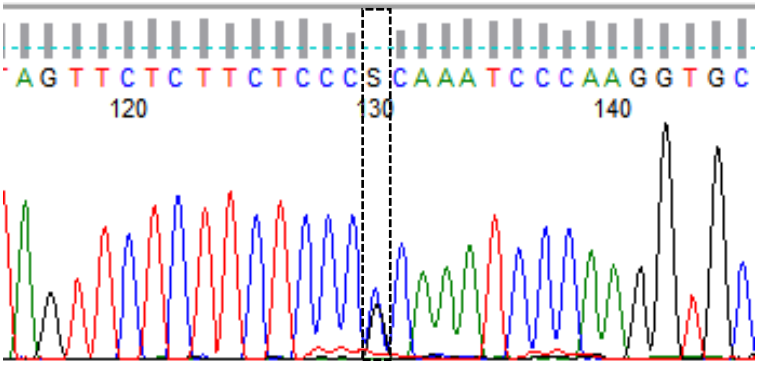

Figure 4.9: Exonic splice silencer g>c -IVS12 on the intronic region prior to exon3 of *ACTG2*; showing a homozygous variant with G and C bases at the same position analysed on FinchTV

These variants were unique and occurred prior to exon 3 and 5 g>c -IVS12 and t>c-IVS3 respectively. The other intronic variant was a splice site acceptor occurring post exon 7 t>c +IVS16 shown in table 4.5. Table 4.6 shows the predicted effects of the variants in the ADL cohort and the exons in which they were observed on. Variants g>c -IVS12 and t>c-IVS3 are splice site donors predicted to be polymorphic variants; which are capable of causing a shift in the splice sites located in these regions. Both g>c -IVS12 and t>c-IVS3intronic SNPs create exonic splice silencers; which inhibit the splicing of introns prior to mRNA translation.

Table 4.5: *ACTG2* variants observed in the ADL cohort, showing the observed variants and the exons they occur in

Sample	Exon3	Exon4	Exon5	Exon6	Exon7	Exon8	Exon9	Exon10
ADL01	WT	WT	c.463 A>G K119R, c.74 t>c-IVS3	WT	c.236 t>c +IVS16	WT	WT	WT
ADL02	c.134 g>c -IVS12	WT	WT	WT	WT	WT	WT	WT
ADL03	c.134 g>c -IVS12	WT	WT	WT	WT	WT	WT	WT
ADL04	c.134 g>c -IVS12	WT	c.463 A>G K119E, c.74 t>c-IVS3	WT	c.236 t>c +IVS16	WT	WT	WT
ADL05	WT	WT	c.463 A>G K119E, c.74 t>c -IVS3	WT	c.236 t>c +IVS16	WT	WT	WT

K119E/R and t>c -IVS3 have the same CADD value 13.43 which places both variants in the 10% of the most deleterious mutations; a prediction score which is in agreement with the other *in silico* analysis predicting the variants to be possibly pathogenic. Although g>c -IVS12 has been predicted to be a polymorphic splice site changing variant; its CADD score is below 1, predicting the variant to be benign. The inverse is true for t>c +IVS16 which is predicted to have no effect by MutationTaster; whereas its scaled CADD score is within the 10% most deleterious mutations.

Table 4.6: *ACTG2* SNPs identified in the ADL cohort, showing the pathogenesis prediction scores of each variant, the exon of occurrence and the number of patients affected

Variant	Exon	MutationTaster	HSF	CADD
K119E	5	-	-	13.43
g>c -IVS12	3	Splice site change	ESS	0.258
c>t -IVS3	5	Splice site change	ESS	13.43
t>c +IVS16	7	No effect	ESS	10.95

ESS = Exonic splice silencer

CHAPTER 5

5. Discussion

5.1.1. Gene expression analysis of HSCR

HSCR is regarded as one of the most common paediatric gastrointestinal disorders affecting the colon. Its aetiology is associated with multiple genes involved in ENS development of which the *RET* proto-oncogene is regarded as the central gene (Carter et al., 2012). Various genes associated with the HSCR phenotype have been discovered through whole exome studies; although for some genes their functional properties have not been validated (Gui et al., 2017). Quantitative studies such as RT-qPCR are essential to provide further insights into the disease mechanism. This study was aimed at providing such a validation of correlating the observed HSCR phenotype and expression of certain genes associated with ENS development, through quantitative analysis of the genes of interest *RET*, *EDNRB*, *NRG1* and *SOX10* shown to have a molecular correlation (Alves et al., 2013). The HSCR study cohort was made up of 13 males and 1 female following one of Hirschsprung's disease characteristics of a male predominance.

Although RNA quality obtained from the study participants was acceptable and the RIN values for the selected samples were well above the minimal 7; the experiment produced low copy number of each sample which resulted in very high C_t values based on the set threshold, these are then classified as non-detects (McCall et al., 2014). These may be caused by other factors including the quality and quantity of the sample RNA and the interference from the SYBR green dye or the primer assays. The selected genes of interest *RET*, *EDNRB*, *NRG1* and *SOX10* each have numerous transcripts of which may promote non-specific binding of the primers in reaction this can be seen by the delayed amplification on the amplification plot. The housekeeping genes (*HPRT* and *HSP*) were also not amplified Primer assays are designed to anneal to the template at certain optimal temperatures and to specific sequences; the possibility of more than one transcript may affect their annealing efficiency of the primer assays.

SYBR green on the other hand is an intercalating dye known to bind to double stranded DNA (Gudnason et al., 2007) with high affinity; should the conversion of RNA to cDNA not occur

efficiently it consequently affect the quantification which may have been the case in this study. In addition, Giglio and colleagues reported that SYBR green has preferential binding to certain DNA sequences (Giglio et al., 2003); which may be the cause of the amplification of some samples over others as shown by the amplification plot . Therefore, a conclusion regarding the correlation between the phenotype and genotype of the commonly reported genes involved in ENS development *RET*, *EDNRB*, *NRG1* and *SOX10* in confirmed HSCR patient was not reached.

5.1.2. HSCR and *ACTG2* genetic factors

Post-operative complications such as bowel obstruction, anastomotic leakage, faecal incontinence and perineal excoriation have been reported in at least 32% of HSCR patients (Jarvi et al., 2010). This evidence suggests that HSCR may be a result of not only ENS pathogenesis; in some affected patients it may be a result of a combination of muscular and neuronal dysfunction. The slow gastric transit experienced by some HSCR patients post resection surgery can be correlated to slow enteric muscular contraction resulting from smooth muscle deficit (Faure et al., 2016). Gastrointestinal motility is a functional consequence of a well-coordinated contraction by muscle layers of the alimentary canal, neuronal current which require an intact neuronal network and muscular layers (Sanders et al., 2012) and the pacemaker ICC.

In this study *ACTG2* which has been associated with the aetiology of visceral myopathy was investigated in patients with histologically confirmed HSCR. Actin forms part of the support and maintenance of the cytoskeleton; these are highly conserved proteins within species as illustrated by the species conservation phylogenetic tree on the appendix figure 7.1. Although actins are found throughout the body, gamma actins are exclusively found in the enteric portion of the body. Through the prospective study of the HSCR cohort the S345L and W357G SNPs were observed in 6 and 1 patient/s respectively; interestingly they were both absent in the control cohort. Both mutations are novel and non-conservative; they were predicted to alter the secondary structure of the ACTG2 protein and are possibly disease causing.

Variants S345L and W357G are categorized by scaled CADD to be in the 1% and 0.1% most deleterious substitutions respectively as shown in table 4.4. As a result, they cannot be overlooked as the essential smooth muscle genetic factors that may contribute to the HSCR pathogenesis. It was further observed that W357G occurred to have bonds with a known VSCM

variant Y134 rs587777388 , which has been identified in numerous familial cases as a *de novo* missense variant (Thorson et al., 2014; Wangler et al., 2014). In addition to the exon 10 variants; another variant was observed in the HSCR cohort occurring on exon 5 K119E/R. K119E/R is also a non-conservative variant; although it was also observed in the healthy control. Consequently, due to the presence of this variant in both the HSCR patients and healthy controls it was deemed as non-significant in disease pathology; however, it may play a role as a disease modifier since the patients that harboured the exon 10 variants S345L and W357G also had the K119E/R variant. The importance of K119E/R is demonstrated by a scaled CADD score of 13.43 as shown in table 4.4; which is within the 10% most deleterious substitutions and can therefore be considered to play a role in disease pathogenesis.

Additionally, other variants namely V153C, V160R, L217A, L243P and intronic variant c>t -IVS6 were observed in the HSCR cohort. However, these were considered as non-significant and not pathogenic based on their pathogenicity score predictors; which were below the value of significance as specified by PolyPhen-2 and CADD. Although V153C and L217A were predicted to be pathogenic by PolyPhen-2 and scaled CADD respectively; they were not investigated further since they were each predicted to be pathogenic by only one platform. The use of *in silico* prediction algorithm to determine whether as SNP is deleterious is dependent on the information available on the tool; mainly the quality of the multiple sequence alignments. It has been reported that most *in silico* prediction algorithms use paralogue protein sequences to predict the effect of amino acid changes instead of a combination of both paralogues and orthologues (Wong and Zhang, 2014). Hence the use of more than one prediction tool is essential in determining the role on the SNP on the protein.

5.1.3. Genetic basis of ADL

Research in HSCR patients focuses solely on factors that control genes involved in ENS development; Gui and colleagues 2017, revealed that ENS genes and variants substantiate only up to 25% of the HSCR genetic risk (Gui et al., 2017). Thus, studying other genes involved in the gastroenteric pathway such as the smooth muscle *ACTG2* may provide relevant information regarding the disease etiopathogenesis. Although the smooth muscles develop and produce slow autonomous contraction independent of the ENS; their development occurs in parallel proving the existence of their poorly understood developmental correlation. This is the first study conducted on this smooth muscle cell gene of HSCR patients and has been submitted to

the Paediatric Surgery International journal; currently under review (Chapter 7, article 2). (Graham et al., 2017)

ADL affects children of Central, Southern and East African descent; it is therefore fitting to be referred to as African degenerative leiomyopathy. ADL forms part of the visceral myopathy spectrum. Unlike VSCM its etiopathology has been linked to environmental factors that deteriorate the muscles of the distal part of the alimentary canal; which leads to an enlarged abdominal area (Rode et al., 1992). Numerous reports of ADL have been isolated; although, RET promoter variants in familial ADL have been observed. The discovery of *RET* variants in a familial ADL study, initiated interest in the prospective study of genes in patients affected with ADL (Van Rensburg et al., 2012). Studying the *ACTG2* gene associated with VSCM which exhibits similar phenotype in affected individuals may provide genetic information for the molecular basis of the disease.

The ADL patients were screened for variants in *ACTG2*. In contrast, the variants identified in the DNA of the study cohort selected for this research did not reveal any of the known VSCM variants. The variants observed were intronic polymorphisms namely g>c -IVS12 on exon 3 and c>t -IVS3 on exon 5; predicted to have pathogenic effect by shifting the exonic splice sites. Furthermore, the c>t -IVS3 of exon 5 co-occurred with the dominant K119E/R variant further suggesting that the variant may have a disease modifying property. Although the variant g>c -IVS12 was predicted to have an effect on the splice site of *ACTG2* prior to exon 3; it was predicted to be tolerated by scaled CADD. The same was observed for the variant t>c +IVS16; which was predicted to no have an effect on the splice site by MutationTaster whereas scaled CADD placed it in the 10% deleterious SNPs. Such data should not be ignored hence the use of more than one prediction tool has proven to be essential; however they did not mitigate the conflicting result. Therefore, further wet bench study of these SNPs is necessary.

This is the first study on the smooth muscle of ADL patients and the data has been published in the Paediatric Surgery International Journal (Chapter 7, article 1). The observation of novel *ACTG2* SNPs in this distinct form of VSCM other than the known variants also suggest that ADL may affect different components of the smooth muscle cells other than the gamma actin cytoskeleton. In conclusion, further investigation of genetic material of patients with histologically confirmed ADL through whole exome sequencing could reveal other contributing genetic factors that lead to its aetiology.

5.1.4. Mutations in HSCR and ADL

Comparing the *ACTG2* SNPs observed in both HSCR and ADL cohorts there are clear differences in the SNPs that were identified within the two cohorts; except the polymorphic variant on the intronic region of exon 3 g>c -IVS12 and the probable disease modifying variant on exon 5 K119E/R which the cohorts shared. This result suggests that HSCR does have a muscular dysfunction component; thus a conclusion may be made that the aetiopathogenesis of HSCR is likely associated with both neuronal and muscular genetic predispositions. Meanwhile ADL can be associated with both genetic and environmental predispositions. *ACTG2* intronic variants (g>c -IVS12 and c>t -IVS3) and coding variants (K119E/R, S345L and W357G) observed in the study were not identified in publicly available domains viz ExAC, 1000 Genome and dbSNP, therefore our variants can be classified as novel.

Studying only the actin genetic factors in these patients does not provide sufficient data regarding the recurrence of the motility deficit in HSCR patients and the genetic basis of ADL pathogenesis. Further study of the myosin *MYH11* in both HSCR and ADL patients may provide information in elucidating the underlying genetic factors that contribute to the risk of recurrence in motility deficit and the molecular cause of ADL; sufficient data may therefore provide a better model for advanced disease management strategies in both pathologies.

5.2. Conclusion

In conclusion, the results obtained in this study suggests that although ENS and smooth muscle development occur in parallel but independent of each other; a temporal correlation does exist which is not yet fully understood. γ- 2 enteric actin encoded by *ACTG2* associated with familial visceral myopathy a known enteric smooth muscle defect; therefore, the data suggests that the HSCR and ADL patients with the novel *ACTG2* variants may have defects in the contractile proteins of their enteric smooth muscles. Taken together these data opens a new field of research into the etiopathology of HSCR which involves the enteric smooth muscle genetic drivers and further provides insights into the genetic mechanism involved in the region specific rare gastrointestinal disease ADL.

5.3. Limitations

The study had a couple of limitation evident in some of the data presented. These include the inability to successfully amplify and consequently prohibit the quantification of the expression of *EDNRB*, *RET*, *SOX10* and *NRG1*; which may also be the result of gradual sample degradation due to repeated freezing and thawing. The use of RNA preserving agents such as RNAlater should be added to the whole blood samples immediately after collection, to aid in the increased yield of extracted RNA. It is known that RNA is easily degradable by RNases that are readily available on most surfaces. Another limitation relates to the sample size; which may be increased and should include sampling from other regions in South Africa; this will enable us to accurately elucidate the incidence of both HSCR and ADL in the South African population. Furthermore, it will provide enough quantity of samples to conduct a comparison of *ACTG2* variants between the cohorts. Most of the data used in this study was generated *in silico* with various prediction and modelling platforms; although these methods are essential in predicting the effects of novel variants since there is no available experimental data for them, they are sometimes unreliable and often require more than one prediction tool to validate the results.

5.4. Future Study

Probes should be considered instead of primer assays and SYBR green for the quantification of *EDNRB*, *RET*, *SOX10* and *NRG1* in order to bypass the disadvantages of using these reagents. In addition, *in vitro* methods such as site directed mutagenesis should be employed to validate the effects of the novel variants K119E/R, S345L and W357G in a model; which is also essential for taking the findings further. Surveillance of HSCR patients throughout their life should be implemented in order to monitor if they experience recurrence of the aganglionosis.

CHAPTER 6

Article 1

Pediatric Surgery International

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Recurrent ACTG2 gene variation in African degenerative visceral leiomyopathy

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Original Article

First Online: 15 November 2018

- 5Downloads

Abstract

Introduction

Visceral myopathies remain difficult and frustrating clinical entities, a distinctive form of acquired degenerative visceral myopathy, African degenerative leiomyopathy, a myogenic functional intestinal obstruction without aganglionosis which affects smooth muscle of the intestine, in young indigenous African children. The Actin G2 gene is the main gene encoding smooth muscle actin found in enteric tissues. Recent research has identified Actin G2 alpha gene variation as an important causative biomarker in visceral myopathies and megacystis microcolon. This study of the Actin G2 gene (ACTG2) in an African population explores a possible molecular basis abnormal muscle function in a visceral myopathy.

Patients and methods

Following ethical permission and informed consent, DNA was extracted from whole blood samples in five patients with histologically proven African degenerative leiomyopathy. PCR amplification of ACTG2 alpha gene products by semi-automated bi-directional sequencing analysis. Results were analysed using FinchTV Sequence Alignment Software and predicting bioinformatic investigation by PolyPhen-2 software.

Results

Five new patients with the ADL phenotypes were prospectively investigated for variation in the Actin G2 gamma gene (ACTG2). ACTG2 gene variation occurred in exon 5 (c.463 A>G K119R), in three (60%). In addition, intronic variation t > c-IVS3 was identified in three with the K119 mutation plus further g> c -IVS12 and t > c + IVS16(2), suggesting a possible haplotype. Bioinformatic modelling showed that these ACTG2 gene variations are highly non-conservative altering protein expression.

Conclusions

Recurrent Actin G2 smooth muscle gene variation in African degenerative visceral leiomyopathy is associated with abnormal muscle actin development.

Keywords

Visceral myopathy African degenerative leiomyopathy ADL Children

Notes

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Article 2

Is Hirschsprung disease a purely neurological condition? A study of the actin G2 smooth muscle gene in Hirschsprung's disease.

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** Address for correspondence Abstract

Hirschsprung disease is a functional obstruction of the gastrointestinal tract due to the congenital absence of ganglion cells in the intermyenteric plexuses of the distal bowel. Gastrointestinal motility requires intact muscular layers as well as neural network connection to function properly. The Actin G2 gene is the main gene encoding actin gamma 2; a smooth muscle actin found in enteric tissues.

This study of the Actin G2 gene in patients with Hirschsprung disease explores a possible molecular basis abnormal muscle function and post-surgical pseudo-obstruction in a group of patients. As far as the authors are aware, this is the first report confirming structural muscle deficits in Hirschsprung disease.

Patients and methods: Ethical permission and informed consent were obtained. DNA was extracted from whole blood samples in 10 patients with histologically proven HSCR patients. PCR amplification of the ACTG2 gene, were subjected to semi-automated bi-directional sequencing analysis. Sequencing results were analysed using FinchTV Sequence Alignment Software (http:/en.biosoft.net) to read chromatogram files. Further predicting bioinformatic investigation was obtained by PolyPhen 2 software to evaluate the significance of the observed amino acid changes.

Results: Ten new patients with similar HSCR phenotypes were prospectively investigated for variation in the Actin G2 gamma gene (ACTG2) variations.

The results of ACTG2 gene analysis showing variation in exons 5, 8 and 10 of the ACTG2 gene in 7 of them (64%). The c.109C>G S345L was the most frequent occurring in 6 of the 10 patients (54%), the c.171 A>A K119E in 2 and the significant c.108 T>G W357G variation in exon 10 (1 patient) Four patients had a combination of different variants in different exons which were less significant.

Allele frequency on a control sample of the South African population showed no comparable pathology link scores (http://gnomad.broadinstitute.org/).

Bioinformatic in silico modelling showed that the residue replacements in both variants (Lys to Glu and Trp to Gly) are highly non-conservative and variation can alter interactions within the protein conformation.

Conclusions: The Actin smooth muscle gene showed variation in 64% of samples, indicating a reason for abnormal functioning muscle in many HSCR patients. Hirschsprungs disease is part of a complex spectrum which also includes smooth muscle.

Key words: Hirschsprung disease; enteric nervous system; smooth muscle; development; ACTG2 gene, mutation; intestinal motility

CHAPTER 7

Appendices

```
Sequence type explicitly set to Protein
Sequence format is Pearson
Sequence 1: sp|P63267|ACTH_HUMAN     376 aa
Sequence 2: sp|P63268|ACTH_MOUSE     376 aa
Sequence 3: tr|F6Z8K0|F6Z8K0_HORSE   376 aa
Sequence 4: tr|G1NTM6|G1NTM6_MYOLU   376 aa
Sequence 5: tr|G3T9R8|G3T9R8_LOXAF   376 aa
Start of Pairwise alignments
Aligning...


Sequences (1:2) Aligned. Score: 100
Sequences (1:3) Aligned. Score: 100
Sequences (1:4) Aligned. Score: 100
Sequences (1:5) Aligned. Score: 100
Sequences (2:3) Aligned. Score: 100
Sequences (2:4) Aligned. Score: 100
Sequences (2:5) Aligned. Score: 100
Sequences (3:4) Aligned. Score: 100
Sequences (3:5) Aligned. Score: 100
Sequences (4:5) Aligned. Score: 100
Guide tree file created:   [clustalw.dnd]
```

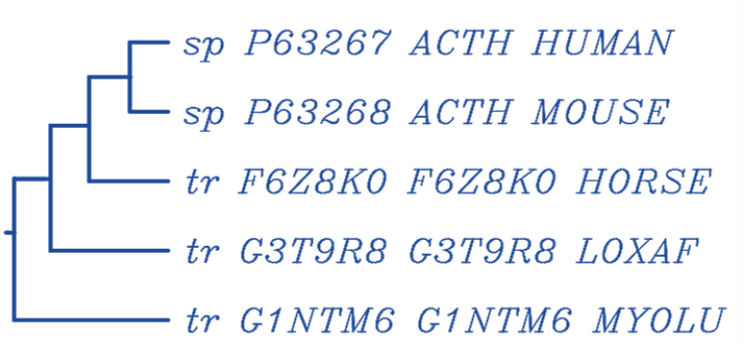

Figure 7.1: Mutiple sequence alignement of the ACTG2 protein of the species: human, mouse, horse, loxaf (Africn elephant) and myolu (little brown bat) generated with CLUSTALW showing the aligment score between the species and a rooted phylogenic tree showing the species conservation of the protein.

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